THE α7β1 INTEGRIN ACCELERATES EXERCISE-INDUCED MYOGENESIS

BY

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THESIS

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ABSTRACT

Muscle-specific transgenic overexpression of the α7β1 integrin can protect against exercise induced-injury and promote the appearance of multipotent mesenchymal-like (Sca-1+CD45-) stem cells in skeletal muscle. **PURPOSE:** The purpose of this study was to determine if overexpression of the α7β1 integrin also results in enhanced and/or accelerated myogenesis following a single bout of eccentric exercise. **METHODS:** 5-wk old, female wild type (WT) and α7 integrin transgenic (α7Tg) mice completed a single bout of downhill running exercise (-20^{o}, 17 m/min, 60 min) and gastrocnemius-soleus complexes were collected 2, 4, and 7 d post-exercise. Myogenesis was quantified using embryonic myosin heavy chain (eMHC) expression and the presence of centrally located nuclei (CLN). **RESULTS:** Whereas gradual increases in eMHC expression were detected in WT mice following exercise, a 5-fold increase in eMHC+ fibers was present at 2 d post-exercise in α7Tg mice compared to α7Tg mice that did not exercise ($P < 0.001$). Consistent with this adaptation, WT mice had gradual increases in numbers of CLN until 7 d post-exercise, whereas α7Tg mice had a 5-fold increase in CLN levels at 2 d post-exercise ($P<0.001$). **CONCLUSION:** This study suggests that the protection from injury and increased appearance of mesenchymal stem cells observed with α7β1 integrin overexpression may directly or indirectly contribute to accelerated myogenesis following eccentric exercise. This study provides the first evidence that the α7β1 integrin participates in myogenesis acceleration.

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CHAPTER I.

INTRODUCTION

A. Purpose

Exercise-induced injury results in the initiation of skeletal muscle regeneration, a relatively slow and highly coordinated process that results in *de novo* synthesis of muscle fibers and the complete repair of muscle tissue. Satellite stem cells residing in the basal lamina are believed to be the primary source of new fiber generation following exercise, yet the precise factors responsible for the activation of these cells are not known. In addition, the role of non-satellite stem cells in the process of myogenesis following exercise-induced (vs. chemical-induced) injury has not been determined. The ability for the α7β1 integrin to enhance skeletal muscle regeneration in a mouse model of muscular dystrophy and the loss of regenerative capabilities in $\alpha7^{-/-}$ mice strongly suggests that this adhesion molecule is a critical factor regulating the regeneration in response to exercise. In addition, suppression of injury and inflammation and the enhanced presence of mesenchymal-like stem cells following exercise in transgenic mice overexpressing α7β1 integrin suggests that there may be numerous ways in which the integrin may increase the extent and rate of myogenesis following exercise. Understanding the molecular mechanisms by which the integrin can enhance regeneration is an important step in revealing its potential in treating a variety of degenerative musculoskeletal conditions. Therefore, the purpose of this study was to determine whether high levels of the α7 integrin in skeletal muscle facilitate the rate and extent of myogenesis following exercise and determine whether mesenchymal-like stem cells have any role in this process.

B. Hypotheses

The presence of the α7β1 integrin in skeletal muscle will suppress injury and inflammation and increase the presence of mesenchymal-like stem cells following a single bout of eccentric exercise such that the following changes will be found:

1. An increase in myogenic markers, embryonic myosin heavy chain (eMHC)+ and the position of nuclei in the center of the fiber (CLN), will be observed in α7 integrin transgenic mice as early as 2 days post-exercise, whereas eMHC+ and CLN+ fibers will gradually increase in wild type mice over a period of 7 days following exercise.

2. The total number of eMHC+ and CLN+ fibers will be higher in α7 integrin transgenic mice compared to wild type mice following exercise.

3. Mesenchymal-like stem cells in muscle of α7 integrin transgenic mice will directly or indirectly contribute to the formation of new fibers following exercise.

4. Enhanced myogenesis will increase function in α7 integrin transgenic mice compared to wild type mice at 7 days post-exercise.

C. Specific Aims

Transgenic and wild type mice will experience a single bout of forced downhill treadmill running at 17 m/min for 1 hour. Mice will be euthanized 2, 4, and 7 days following exercise and myogenesis will be measured using a variety of immunohistochemistry techniques. Functional muscle force responses will be measured using *in situ* stimulation of the sciatic nerve in a separate set of mice.

1. The extent of embryonic myosin heavy chain expression, Pax7 expression, and the presence of centrally located nuclei will be measured and compared between WT and α7 integrin transgenic mice. **(Hypothesis #1)**

2. The rate of embryonic myosin heavy chain expression, Pax7 expression, and the presence of centrally located nuclei will be measured and compared between WT and α7 integrin transgenic mice. **(Hypothesis #2)**

3. Using previously collected samples, the expression of eMHC will be measured in Sca-1+CD45- mesenchymal-like stem cells which were labeled with a fluorescent dye for tracking purposes and injected into mice following exercise. In addition, Pax7 expression in both endogenous satellite stem cells and the injected Sca-1+CD45-mesenchymal-like stem cells will be measured and compared to contralateral control muscle that did not receive the injected cells. **(Hypothesis #3)**

4. Muscle force will be measured and compared between WT and α7 integrin transgenic mice at 7 days post-exercise using *in situ* stimulation of the sciatic nerve in a separate set of mice. **(Hypothesis #4)**

D. Definition of Terms

Transgenic refers to the genotype of the genetically-altered mice. These mice possess copies of an exogenous α7 integrin gene fused with a creatine kinase (MCK) promoter, allowing expression of the α7β1 Integrin 8 times greater than wild type mice in skeletal muscle (Boppart et al., 2006).

Myogenesis is the coordinated process of new fiber formation which occurs during development or as a result of injury and the process of regeneration. Myogenesis (hyperplasia)

and enlargement of existing fibers (hypertrophy) are responsible for increases in mass and function that are observed following exercise. Regeneration is the recovery process during which skeletal muscle replaces damaged or necrotic tissue following injury. Myofiber injury results in necrotic fibers, thereby activating an inflammatory response (Tidball et al., 2005) which is followed by a regenerative state, in which satellite cells (resident muscle stem cells) are activated (Karpati et al., 1988), centrally located nuclei are present, and regenerating fibers appear (Karpati and Molnar, 2008). Newly generated fibers, not produced in the basal lamina of pre-existing fibers, but originating separately from stem cells, are termed "*de novo* muscle fibers".

Multipotent mesenchymal-like stem cells are undifferentiated cells that are capable of giving rise to a limited number of different cell types. A multipotency characteristic of a cell refers to its ability to differentiate into multiple lineages. Mesenchymal progenitors specifically give rise to muscle, bone, tendon, ligament, and fat cells (Oreffo et al., 2005).

Satellite cells are a quiescent population of undifferentiated progenitor cells, often termed stem cells, that are resident to the muscle fiber between the basal lamina and the sarcolemma (Watt & Hogan, 2000). Satellite cells remain quiescent until they become activated by the appropriate stimuli. When activated, they participate in tissue regeneration and repair and are the primary contributors to growth, remodeling, and regeneration (Carosio, Berardinelli, Aucello, & Musaro, 2009).

Mechanotransduction refers to the mechanism by which mechanical strain on the muscle cells is converted into chemical activity in response to that strain. As force is eccentrically loaded on the skeletal muscle of the mice, a mechanical strain is induced which translates into both intra- and inter-cellular chemical signaling.

CHAPTER II.

LITERATURE REVIEW

A. α7β1 Integrin

The term integrin refers to the function of "integrating" the exterior (the extracellular matrix) and interior (cytoskeleton) environments of a cell. Integrins are heterodimeric glycoproteins comprised of non-covalently bound α and β subunits that link extracellular matrix ligands with actin in the cytoskeleton (Song, Wang, Foster, Bielser, & Kaufman, 1992) (FIGURE 1). At least 18 α subunits and 8 β subunits have been characterized, resulting in the generation of 24 unique possible integrin heterodimers (Song, Wang, Sato, Bielser, & Kaufman, 1993). Splicing of α7 transcripts produces alternative cytoplasmic (α7A and α7B) and extracellular domain variants (X1 and X2) with the α7BX2 being the dominant heterodimer in proliferating myoblasts and adult skeletal muscle (Burkin & Kaufman, 1999) (FIGURE 2). Unlike the α7B integrin isoform, which is expressed in a variety of cell types, expression of the α7A integrin isoform is restricted to skeletal muscle and found in mature muscle following myogenesis. Skeletal muscle also coordinates the developmental expression of extracellular splice variants, with X1 predominant during myoblast differentiation and X2 preferentially present in adult fibers. (Schober et al., 2000). These different α7β1 heterodimers localize to specific sites on myofibers and appear to have distinct functions in skeletal muscle, yet most data on α7β1 function *in vivo* are limited to the α7BX2 isoform or do not address this issue.

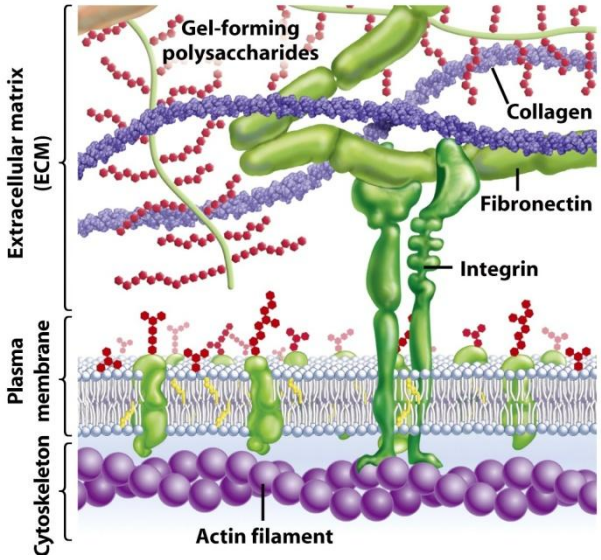


FIGURE 1. Integrins are a family of transmembrane glycoproteins that link the extracellular matrix with actin in the cytoskeleton (Freeman, 2005).

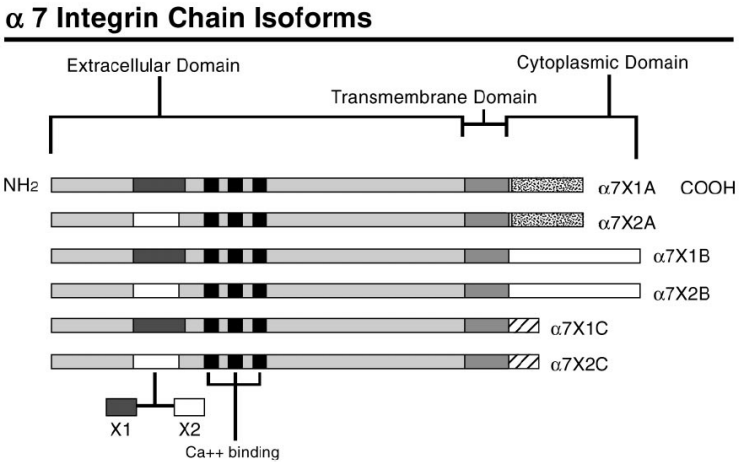


FIGURE 2. Chain isoforms of the α7 integrin (Burkin & Kaufman, 1999).

B. Role of the α7β1 Integrin as a Mechanotransducer that Regulates Intracellular Signaling in Skeletal Muscle

The α7β1 integrin appears to be critical for skeletal muscle development and contractile function as it is present in myoblasts during myogenesis, expressed throughout the sarcolemma and in Z bands in adult fibers, and enriched at myotendinous (Bao, Lakonishok, Kaufman, & Horwitz, 1993) and neuromuscular (Martin, 2003) junctions. A critical role for the α7β1 integrin in skeletal muscle can be derived from genetic studies demonstrating that mutations in the β1

integrin gene are lethal in embryonic mice and mutations in the α7 gene (ITGA7) result in human congenital myopathies (Hayashi et al., 1998) and progressive muscular dystrophy in mice (Mayer, 2003). The α7 integrin subunit specifically binds laminin in the basal lamina surrounding individual muscle fibers, while the β1 subunit attaches to the actin cytoskeleton within the cell (Boppart, Volker, Alexander, Burkin, & Kaufman, 2008) (FIGURE 3). Thus, a primary role of α7β1 integrin in skeletal muscle is to relay information regarding alterations in mechanical forces from the outside-in and inside-out. The α7β1 integrin may also act as a scaffold protein within costameres and myotendinous junctions, providing protection from high forces that occur during lengthening contractions (Boppart et al., 2008).

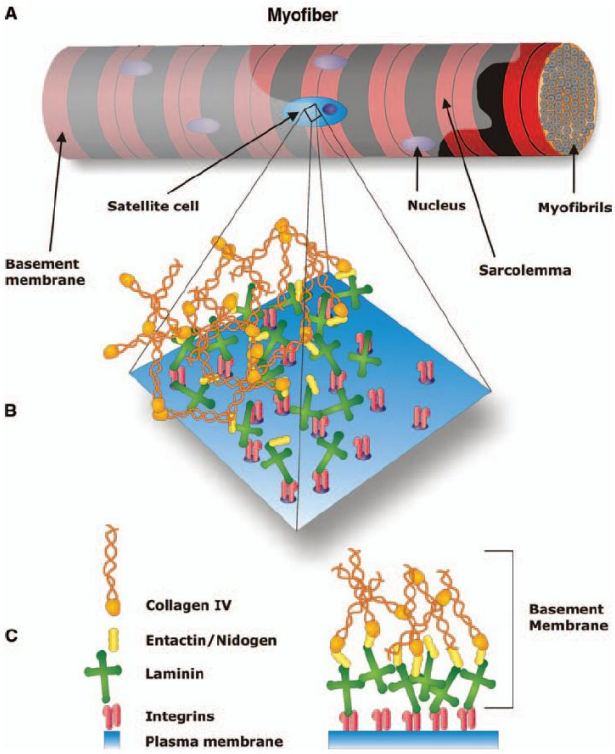


FIGURE 3. Coupling of the basement membrane to the sarcolemma in skeletal muscle (Boonen & Post, 2008).

It has been proposed that the α7β1 integrin can act as a mechanotransducer of cellular signaling and alter biologic activities and gene expression by initiating protein interactions and phosphorylation events within muscle fibers. The cytoplasmic domain of the α7B isoform is composed of 77 amino acids, the largest of all integrin α-chains (Von der Mark et al., 1991), and amino acid sequences homologous with other receptors suggest potential for protein binding and subsequent signaling events (Song et al., 1992; Song et al., 1993). In addition, the associating β1 integrin subunit binds numerous intracellular signaling molecules and clearly stimulates and/or modulates downstream signaling cascades (Hannigan et al., 1996; Ffrench-Constant & Colognato, 2004; von der Mark et al., 1991). Unfortunately, it is difficult to distinguish α7- and β1- integrin subunit-specific roles in skeletal muscle signaling since both must be present for proper heterodimer function. Despite this limitation, Boppart, Burkin, and Kaufman (2006) have demonstrated that intracellular signaling is markedly downregulated following eccentric exercise in mouse skeletal muscle. It is clear that the α7β1 integrin modulates intracellular protein function, yet detailed information regarding these interactions is not known.

C. α7β1 Integrin and Disease

Skeletal muscle serves as the source of power for physical activities essential for survival including respiration and locomotion (Choi et al., 2005). Skeletal muscle atrophy is a broad term that encompasses several diseases and conditions resulting in decreases in muscle mass, strength, and most importantly, function. Muscle atrophy is associated with infectious (septicemia, pneumonia) and noninfectious (cardio-respiratory insufficiency) complications while severe muscle atrophy is associated with an increase in mortality (Kotler, 2000). Whether atrophy occurs rapidly as demonstrated in cancer cachexia, or slowly as observed with age-related sarcopenia, muscle loss increases morbidity and mortality and provides a substantial financial

burden to the United States' healthcare system. It is estimated that the healthcare expenditures attributable to sarcopenia alone are $18 billion per year (Kotler, 2000) and currently available treatment strategies are often inadequate (Janssen, Shapard, Katzmarzyk, & Roubenoff, 2004). Discoveries made in recent years emphasize that integrins are indispensable both for muscle development and muscle function in adult skeletal muscle (Mayer, 2003) and may have a role in combating myopathies (Burkin et al., 2005; Burkin, Wallace, Nicol, Kaufman, & Kaufman, 2001).

Muscular dystrophy is categorized by the absence or deficiency of the dystrophin protein used as the scaffolding of the muscle fiber. Patients with Duchenne muscular dystrophy, and *mdx* mice that also lack dystrophin, exhibit increased levels of α7β1-integrin (Hodges et al., 1997). Without dystrophin, the muscle fibers lose their structural integrity and weaken, resulting in loss of contractile function. Therefore, it is suggested that the increase in the α7β1 linkage of the extracellular matrix and myofibers may compensate for the absence of the dystrophin (Burkin & Kaufman, 1999). Muscle-specific transgenic overexpression of the α7BX2 integrin has been shown to ameliorate loss of muscle structure, increase function, and extend longevity in a severely dystrophic mouse model (Burkin et al., 2001). Thus, these studies suggest that increased α7BX2 integrin may have therapeutic potential for a variety of myopathies and sarcopenia. It will be important to determine the mechanism of integrin action prior to use as a clinical tool. While its primary role may be to ensure a stable mechanical link between the muscle fiber and the extracellular matrix, it also has been suggested that the α7β1 integrin may promote muscle regeneration by increasing muscle stem cell recruitment and proliferation (Burkin & Kaufman, 1999; Rooney et al., 2009).

D. Skeletal Muscle Regeneration

1. Overview of Skeletal Muscle Regeneration

There are four interrelated and time-dependent steps involved in muscle regeneration and repair. These are: degeneration, inflammation, regeneration, and remodeling-repair (Crisco, Jokl, Heinen, Connell, & Panjabi, 1994) (FIGURE 4). Injury to myofibers, resulting from exercise, disease, or acute injury, causes a rapid state of necrosis. This is due to an influx of extracellular calcium, inducing proteolysis of the myofibers (Oberc & Engel, 1977; Bodensteiner & Engel, 1978). An inflammatory response is then activated due to the presence of necrotic fibers. The muscle inflammatory response is characterized by the sequential invasion of specific inflammatory cells (Tidball, 2005). Following the inflammatory response, the regenerative phase is characterized by satellite cell activation and the presence of regenerating fibers with centrally-located nuclei (Karpati and Molnar, 2008). Satellite cells and/or their progeny fuse with existing myofibers or fuse with each other to form new myofibers (Grounds & Yablonka-Reufeni, 1993; Hawke & Garry, 2001). The remodeling-repair stage involves the maturation of the fully regenerated fibers, the remodeling of the extracellular matrix, and the recovery of functional performance (Goetsch, Hawke, Gallardo, Richardson, & Garry, 2003). Since subtle myofiber injuries routinely occur during normal muscle activity, the need for ongoing repair is essential for muscle maintenance (Shefer, Van de Mark, Richardson, & Yablonka-Reuveni, 2006).

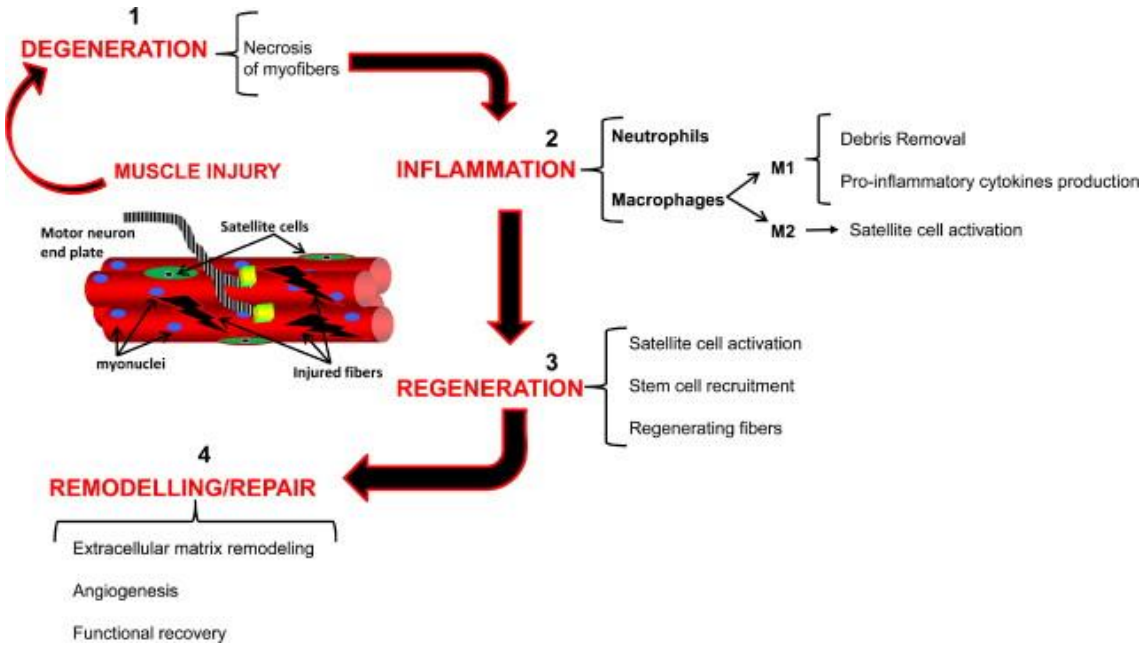


FIGURE 4. The four steps of skeletal muscle regeneration and repair (Carosio et al., 2009).

1.1 Inflammation

Inflammation is a critical component of the regenerative process. The first inflammatory cells to infiltrate the injury site are the neutrophils, which remove cellular debris by releasing free radicals and proteases and stimulate the homing of macrophages by secreting pro-inflammatory cytokines (Tidball, 2005). Macrophages, the predominant inflammatory cell type, are thought to have both a direct (removing tissue debris) and indirect (activation of stem cells) role in mediating muscle regeneration (Arnold et al., 2007). M1 macrophages are the acute responders to injury, producing pro-inflammatory cytokines and releasing oxygen-free radicals (Krippendorf & Riley, 1993; Nguyen & Tidball, 2003; St. Pierre & Tidball, 1994). Conversely, M2 macrophages infiltrate later, scavenging debris and promoting angiogenesis and tissue repair (Krippendorf & Riley, 1993; St. Pierre & Tidball, 1994; Tidball & Wehling-Henricks, 2007). M2 macrophages have the ability to release cytokines and growth factors that activate satellite cells

and stem cell populations (Merly, Lescaudron, Rouaud, Crossin, & Gardahaut, 1999). Although the inflammatory process is deemed necessary for regeneration, a prolonged inflammatory response may actually delay the healing process in skeletal muscle characteristic of the disease process or following extreme injury.

1.2 Satellite cells

The satellite cell is an undifferentiated muscle precursor cell located between the sarcolemma (muscle fiber membrane) and basal lamina of the fiber (Campion, 1984). During adult life there is low myonuclear turnover, with only a sporadic requirement for hypertrophy or repair, so satellite cells are mitotically quiescent (Gnocchi, White, Ono, Ellis, & Zammit, 2009). When activated by a variety of muscle-, vessel-, and inflammatory cell-derived growth factors in response to injury, they participate in tissue regeneration and repair and are the primary contributors to growth, remodeling, and regeneration (Carosio, Berardinelli, Aucello, & Musaro, 2009; Kadi et al., 2005). Once activated, satellite cells enter into the cell cycle and express relevant markers such as desmin, myf-5, and MyoD (Yablonka-Reuveni & Rivera, 1994; Zammit, 2008). The transition from cell proliferation to terminal differentiation involves the inhibition of cell division and activation of markers such as embryonic myosin heavy chain (eMHC) and myogenin and the upregulation of Pax7 (Yan et al., 2003) (FIGURE 5).

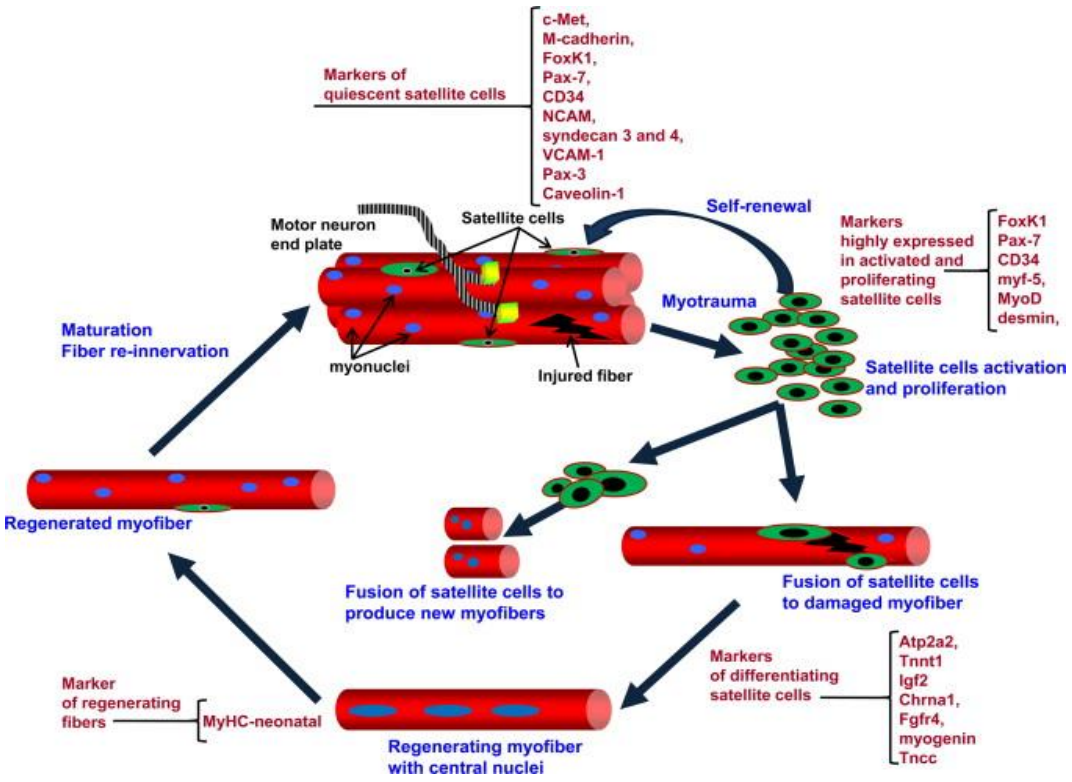


FIGURE 5. Different stages of stem-cell mediated muscle regeneration (Carosio et al., 2009).

Traditionally, satellite cells were considered to be a homogeneous population of committed muscle progenitors. However, many studies have suggested that satellite cells are a heterogeneous mixture of stem cells and committed myogenic progenitors. As a tissue-specific stem cell population, the satellite cell must carry out two roles: it must provide differentiating progeny that will fuse with both new and damaged myofibers and also repopulate the stem cell niche through a self-renewal mechanism. These processes require one of two possible pathways to occur. Either a subpopulation of the satellite cell progeny must stop itself from progressing down the myogenic lineage and revert back to a quiescent state, or a subpopulation of the satellite cells must divide asymmetrically, thus forming both differentiating and stem cell progeny (Carosio et al., 2009). In particular, Kuang, Kuroda, Le Grand, and Rudnicki (2007) demonstrated that Pax7+ satellite cells that lack Myf5 expression have a “stem-like” phenotype

within the satellite cell population. In contrast, Pax7+ satellite cells expressing Myf5 were shown to be more committed to a muscle fate (Kuang et al., 2007).

Aside from their primary role in adult muscle fiber repair, satellite cells are also responsible for postnatal growth (Seale et al., 2000) and hypertrophy of skeletal muscle (Grounds & Yablonka-Reufeni, 1993). It is believed that the restricted tissue repair program under pathological conditions is due to either a loss or shortage of stem cell populations (such as satellite cells), or to missing signals which limits the damaged tissue to efficiently activate a regenerative program (Carosio et al., 2009).

1.3 Niche

The microenvironment "niche" within skeletal muscle refers to the direct exposure of muscle stem cells to specific extracellular components, hormones and growth factors, which directly regulate their proliferation, migration, differentiation, and self-renewal (Kuang, Charge, & Seale, 2006; Sanes, 2003). For satellite cells, contact with a specific niche found between the sarcolemma and the basal lamina is crucial for the suppression of cell division and activation of the intrinsically programmed myogenic cascade. Loss of contact with the niche results in decreased self-renewal and a return to quiescence (Otto, Collins-Hooper, & Patel, 2009). Throughout the regeneration process, the pre-existing basal lamina is believed to serve as scaffolding for the formation of the new fiber and is thought to provide a favorable local environment (niche) for the regeneration (Armand et al., 2003) (FIGURE 6). Preservation of the basal lamina also plays an important role in guiding regenerating motor axons to the original neuromuscular endplate (Bodine-Fowler, 1994).

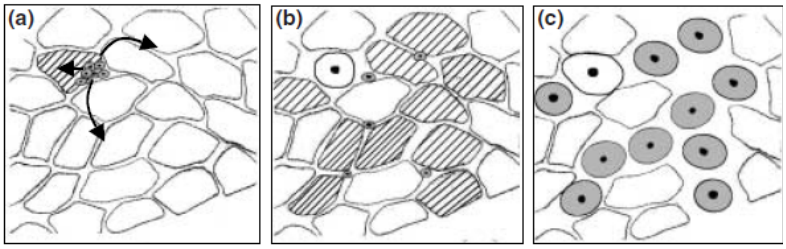


FIGURE 6. A. Clusters of activated satellite cells begin the regeneration process in existing fibers which provide a favorable niche for differentiation and **B.** subsequent fusion with existing fibers to form **C.** new myofibers (Armand et al., 2003).

1.4 Remodeling and Repair

It has been demonstrated that, upon muscle injury, fibroblasts within muscle interstitial tissue are stimulated to produce several types of collagens, contributing to the formation of scar tissue (Mutsaers, Bishop, McGrouther, & Laurent, 1997). This fibrotic response is initially beneficial to the tissue as it is rapid, adds support for strength, and helps in protecting the injury site. However, this overproduction of collagens often leads to heavy scarring and a loss of muscular function (Lehto, Jarvinen, & Nelimarkka, 1986). Therefore, the reparative process is considered complete once the functional performance is restored to the injured myofibers (Pelosi et al., 2007) (FIGURE 4).

1.5 Exercise and Regeneration

Extensive research in recent years has significantly improved our understanding of exercise-induced skeletal muscle adaptation. It is now believed that an orchestrated transduction of signals from neuromuscular activity, mechanical stress, and metabolic factors (Baldwin & Haddad, 2002) to the regulatory machinery of the genes in the mature myofibers plays a central role in mediating skeletal muscle adaptation (Choi et al., 2005). It is well documented that the strain associated with a single bout of downhill running exercise in untrained individuals can induce a physiological amount of skeletal muscle injury in humans (as assessed by elevations in

creatine kinase and deficits in force). This injury response initiates a repair process, which includes production of pro- and anti-inflammatory cytokines, increased satellite cell proliferation, and enhanced protection from future injury.

2. Role of Non-Satellite Stem Cells in Muscle Regeneration

2.1 Non-satellite Stem Cells Express Sca-1+

Sca-1 is a member of the Ly-6 multigene family encoding a number of highly homologous, glycosyl-phosphatidylinositol (GPI)-anchored surface membrane proteins, and is expressed on the surface of non-satellite stem cells, including side population (SP) cells, muscle-derived stem cells (MDSC) (Asakura, Seale, Girgis-Gabardo, & Rudnicki, 2002; Jankowski, Deasy, Cao, Gates, & Huard, 2002) and other myogenic precursor cells (MPCs) recruited to sites of skeletal or cardiac muscle injury (Asakura et al., 2002; Polesskaya, Seale, & Rudnicki, 2003). Sca-1 protein in these cells functions as a regulator of myogenic repair *in vivo* through its control of MPC proliferation and regulates the tempo of muscle repair by controlling the balance between proliferation and differentiation of activated MPCs (Epting et al., 2008).

Kafadar et al. (2009) showed that Sca-1 is upregulated in a subset of myogenic cells in response to skeletal muscle injury. Epting et al. (2008) determined that Sca-1 expression was temporally downregulated on α7+ myoblasts during *in vivo* differentiation. Furthermore, Sca-1 -/- myoblasts exhibit delayed cellular differentiation *in vivo* and Sca-1 -/- knockout mice are known to exhibit delayed skeletal muscle repair following injury (Epting et al., 2008). These results support the belief that Sca-1 functions as a regulator of myogenic repair *in vivo* through its control of myoblast proliferation.

It has been suggested that one of the functions of Sca-1 in MPCs following injury is to upregulate the activity of matrix metalloproteinases (MMPs), thereby promoting the breakdown of the ECM and facilitating normal regeneration by removing ECM components and providing space for new fibers to form or releasing growth factors from the ECM, facilitating new fiber growth (Kafadar et al., 2009). Progenitor cells from Sca-1−/− mice display a homing defect, suggesting that another function of Sca-1 may its involvement in the homing of MPCs to sites of injury (Bradfute, Graubert, & Goodell, 2005).

2.2 Side Population (SP) Cells (Sca+CD45-)

The role of the satellite cell as the predominant muscle progenitor has been challenged following the discovery of several myogenic stem cell populations residing in muscle. It has been suggested that these newly revealed stem cell populations can reside in distal locations such, as within bone marrow, and arrive via the circulatory system in response to homing signals emitted by muscle or simply reside in skeletal muscle tissue and proliferate in response to injury (Carosio et al., 2009). Side population cells are multipotent stem cells residing in bone marrow and skeletal muscle that have the ability to reconstitute bone marrow in lethally irradiated mice. SP cells can enter the satellite cell niche with varying efficiencies in regenerating skeletal muscle (Asakura et al., 2002; Dellavalle et al., 2007; Gussoni et al., 1999; LaBarge & Blau, 2002; Polesskaya et al. 2003; Sampaolesi, Blot, & D'Antona, 2006). It is possible that SP cells from the bone marrow may leave the vessel wall, enter the interstitial space, and cross the basal lamina of the muscle fiber, eventually adopting a satellite cell position and possibly expressing satellite cell specific genes (Messina, Biressi, & Cossu, 2008). Skeletal muscle SP cells can also incorporate into *mdx* regenerating myofibers and restore dystrophin expression (Asakura et al., 2002; Bachrach et al., 2004; Gussoni et al., 1999). Tanaka, Hall, and Troy (2009) have shown

that SP cells, identified through Sca-1 and the ATP-binding cassette ABCG2, contain a subpopulation that also co-expresses syndecan-4, a marker for all satellite cells.

Unlike bone marrow SP cells, skeletal muscle SP cells do not express CD45, allowing one population to be distinguished from another (Gussoni et al., 1999; Montanaro, Liadaki, Schienda, 2004; Rivier et al., 2004). Skeletal muscle SP cells reside in the interstitial connective tissue of the skeletal muscle juxtaposed to blood vessels, express no myogenic markers and do not spontaneously give rise to muscle *in vitro*. However, these cells express Pax7 and myosin heavy chain when co-cultured with C2C12 myoblasts, suggesting they do have myogenic capacity (Asakura et al., 2002; Gussoni et al., 1999).

Skeletal muscle SP cell fractions are heterogeneous and contain at least three subpopulations: CD31+ CD45- SP cells, CD31-CD45+SP cells, and CD31- CD45-SP cells (Uezumi et al., 2006). CD31+ CD45- SP cells (more than 90% of all SP cells in normal skeletal muscle) are associated with the vascular endothelium and do not proliferate following cardiotoxin-induced muscle injury (Uezumi et al., 2006). CD31-CD45+ SP cells are hematopoietic, migrate to injured muscle from bone marrow and are believed to participate in fiber formation (Ojima et al., 2004). Although CD31-CD45- SP cells constitute only 5 to 6% of all healthy adult skeletal muscle SP cells, they have been shown to actively expand during the early stages of muscle regeneration and return to baseline levels following the completion of muscle regeneration (Uezumi et al., 2006).

2.3 Mesenchymal Stem Cells (Sca+CD45-)

CD31-CD45- side population cells have been shown to be of mesenchymal lineage, having the ability to differentiate into adipocytes, osteogenic cells, and muscle cells following specific *in vitro* induction (Uezumi et al., 2006). These particular SP cells are resident in skeletal

muscle and proliferate vigorously in the interstitium. In their 2008 study, Motohashi et al. demonstrated that these cells do not directly become myogenic following transplantation into muscle. Rather, these cells dramatically improve myoblast transplantation when co-injected into both healthy, wild type and dystrophin-deficient *mdx* mice. (Motohashi et al., 2008).

Several researchers have reported that mesenchymal stem cells (MSCs) secrete a variety of cytokines and growth factors that suppress the local immune system, inhibit fibrosis and apoptosis, enhance angiogenesis, and stimulate mitosis and differentiation of tissue-specific stem cells (Caplan & Dennis, 2006). Since CD31-CD45- SP cells are found in close vicinity to myoblasts 48 hours following transplantation, even low levels of growth factors produced by CD31- CD45- SP cells may effectively stimulate the proliferation of myoblasts (Motohashi et al., 2008).

E. Role of the α7β1 Integrin in Skeletal Muscle Regeneration

1. Evidence Suggesting a Role for the α7β1 Integrin in Regeneration with Disease

Enhanced expression of the α7 integrin in the skeletal muscle of mice with severe muscle disease results in an increase in the number of satellite cells in muscle and the fusion of these cells into fibers during repair (Burkin et al., 2005) (FIGURE 7). Inflammation as assessed by hematoxylin and eosin staining is also decreased in dystrophic mice overexpressing the α7 integrin (Burkin et al., 2005). Rooney, Gurpur, Yablonka-Reuveni, and Burkin (2009) demonstrated that there is delayed regeneration in α7-/- mice. These data suggest that the α7BX2β1 integrin may be instrumental in promoting muscle regeneration during disease by increasing muscle stem cell proliferation and/or altering the cytokine microenvironment.

Similarly, the role of the α7BX2β1 integrin in regeneration following exercise-induced injury is not known and is the central theme of this thesis.

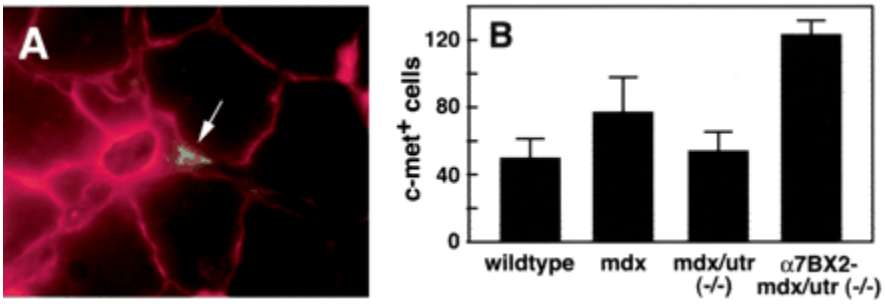


FIGURE 7: α7BX2β1 transgenic mice have increased satellite cell numbers. **A.** An antibody against c-met (hepatocyte growth factor) was used to identify satellite cells. c-met+ cells (green) were scored with wheat germ agglutinin staining used to delineate muscle fibers (red). **B.** A two-fold increase in satellite cells was detected in the muscle of α7BX2β1 transgenic mice compared to non-transgenic animals (Burkin et al., 2005).

2. Evidence Suggesting a Role for the α7β1 Integrin in Exercise-Induced Myogenesis

2.1 The α7 Integrin is Upregulated in Wild Type Mice in Response to Exercise-Induced Muscle Damage and Localizes to Myotendinous Regions

Boppart et al. (2006) and Boppart et al. (2008) previously demonstrated that RNA transcripts of several α7 integrin isoforms, predominantly α7A and α7X1, are increased in wild type mice 3 hrs post-exercise and total α7 integrin protein is increased particularly at myotendinous junctions 24 hrs post-exercise, remaining elevated for one week (FIGURE 8). Transcription of other integrin subunits known to be expressed within muscle remained unaltered, suggesting the α7 integrin subunit is uniquely sensitive to an exercise stimulus.

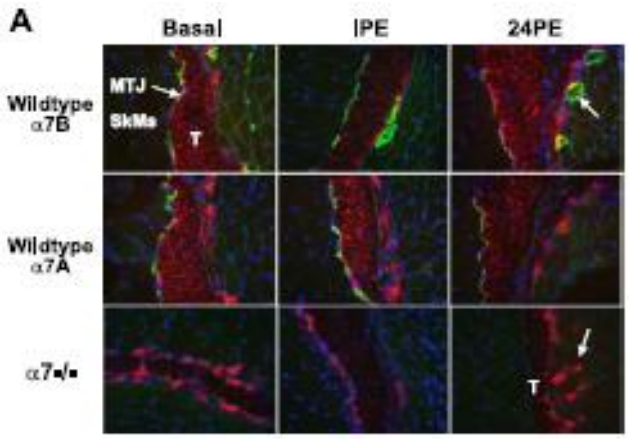


FIGURE 8. The α7 integrin localizes to myotendinous regions at 24 hours post-exercise and remains elevated for one week **(**Boppart et al., 2008).

2.2 α7β1 Integrin Overexpression Confers Protection and Reduces Inflammation in the Muscle while $\alpha7^{-/-}$ Mice Have Pronounced Exercise-Induced Damage

Overexpressing the α7β1 integrin significantly protects skeletal muscle from exercise-induced injury (Boppart et al., 2006; Boppart et al., 2008). Transgenic mice overexpressing the α7 integrin (α7Tg) demonstrated significantly reduced levels of injury as assessed by Evans blue dye incorporation into muscle fibers in which the sarcolemma has been compromised (FIGURE 9). Likewise, deletion of the α7 integrin in α7-/- mice exacerbates muscle damage in response to downhill exercise, especially in the myotendinous junction areas which are exposed to high forces during exercise (Boppart et al., 2008). α7-/- knockout mice also exhibit defective skeletal muscle regeneration following this pronounced exercise-induced injury (Rooney et al., 2009).

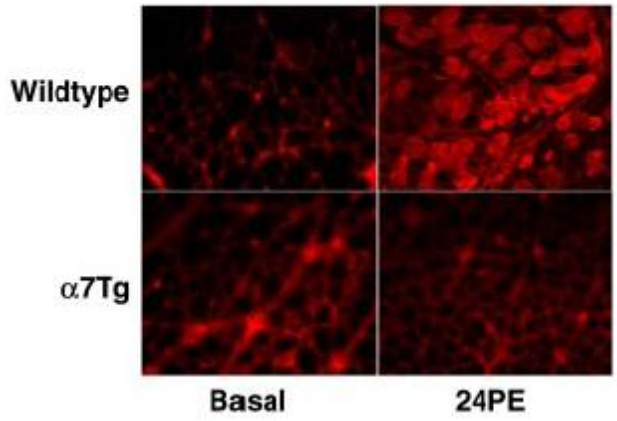


FIGURE 9. Evans Blue Dye incorporation into the fibers of wild type mice 24 hours following a strenuous bout of exercise while the α7Tg mice show protection from injury (Boppart et al., 2006).

It has been shown that suppression of the inflammatory response results in the stabilization of muscle strength and function in muscular dystrophy patients (Manzur, Kuntzer, Pike, & Swan, 2004). α7Tg mice have decreased inflammation following injury (Boppart, publication in progress) (FIGURE 10). The inflammatory response is measured by the concentration of macrophage infiltration to the site of tissue injury. Using F4/80 as a macrophage marker, much lower levels of F4/80+ mononuclear cells were observed in the α7Tg mice following exercise. In addition, a qRT-PCR array demonstrated a 2-fold decrease in IFN-γ (pro-inflammatory cytokine) and a 4-fold increase in IL-13 (anti-inflammatory cytokine) (Boppart, publication in progress) (FIGURE 11). These changes are likely due to lack of injury in response to exercise.

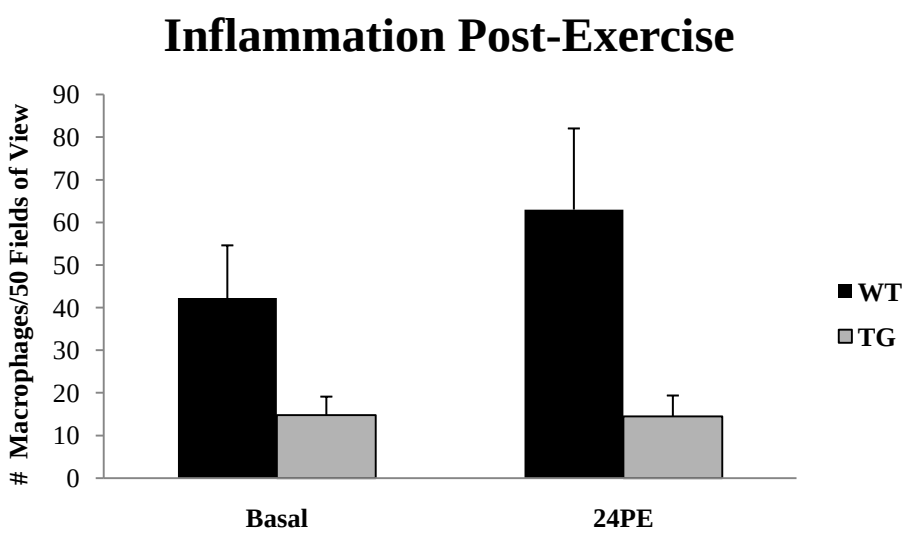


FIGURE 10. Measuring the concentration of macrophage infiltration (F4/80 staining) demonstrated that the α7Tg animals have decreased inflammation both at baseline and following exercise (Boppart, publication in progress).

Cytokine	Fold-Change in α7Tg 24 h PE over WT Basal
IFN-γ	-2.3
IL-11	-2.8
IL-17b	-4.6
IL-13	+3.9
IL-12b	+6.9
IL-24	+2.1

FIGURE 11. Quantitative Real-Time PCR Array data demonstrating the fold changes in both pro- and anti-inflammatory cytokines in the α7Tg mice over wild type mice at 24 hours post-exercise (Boppart, publication in progress).

2.3 The α7β1 Integrin is Prerequisite for the Appearance of Sca-1+ Cells in Skeletal Muscle

Preliminary data collected in the Boppart Lab demonstrate that α7Tg mice acquire elevated levels of Sca-1+ mononuclear cells in the basal state and that these cells are enhanced following an acute bout of eccentric exercise in several areas of the muscle, including the interstitium, myotendinous junction, and vessels-nerve bundles (Boppart, publication in progress) (FIGURE 12). Further characterization using PCR and immunohistochemistry methods reveal that these cells are Sca-1+ CD45- CD31- and express several mesenchymal stem cell markers, suggesting that these cells may enhance the process of regeneration process following injury (Boppart, publication in progress) (FIGURE 13).

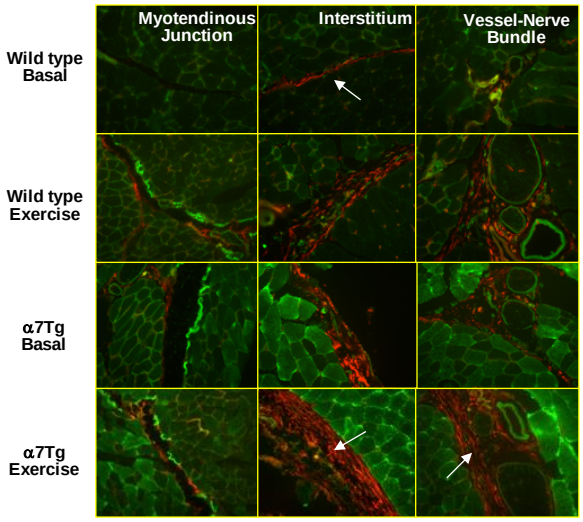
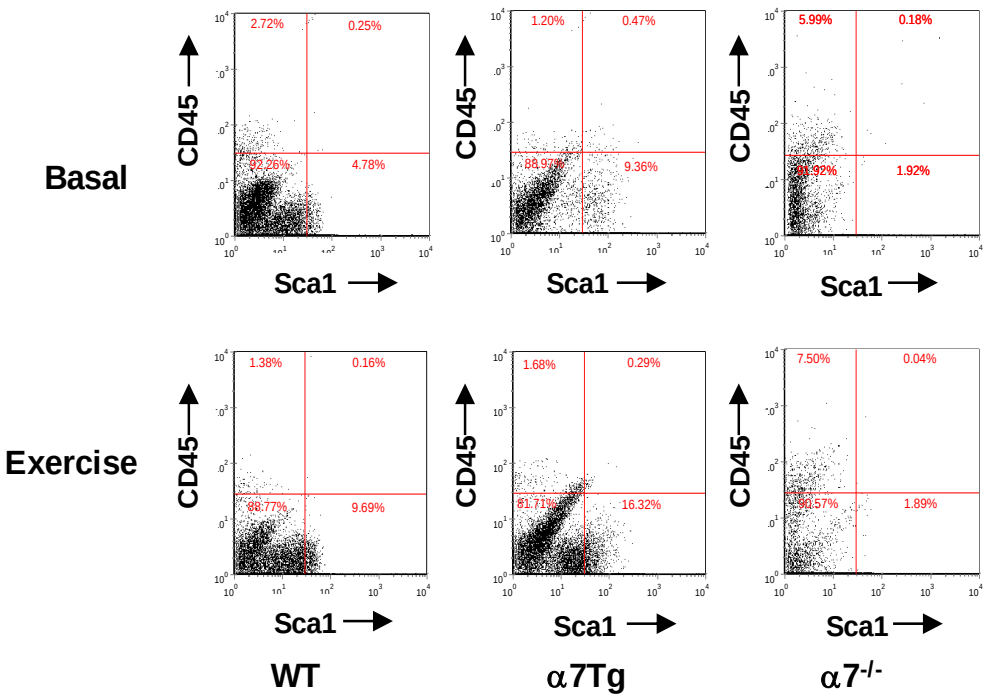


FIGURE 12. α7Tg mice have elevated levels of Sca-1+ mononuclear cells in the basal state which is exacerbated with an acute bout of eccentric exercise in the interstitium, myotendinous junction, and vessels-nerve bundles (Boppart, publication in progress).

A.



B.

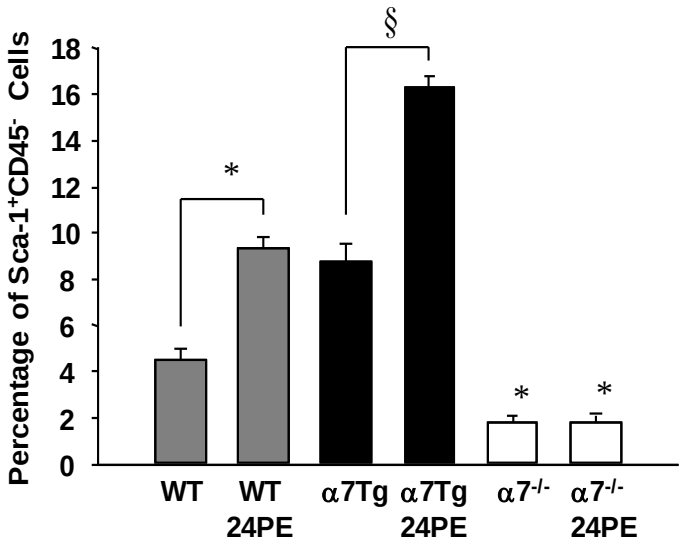


FIGURE 13. A. Characterization of the Sca-1+ mononuclear cells by flow cytometry. **B.** The percentage of Sca-1+ CD45- cells in wild type, α7Tg, and α7 knockout mice at 24 hours post-exercise (Boppart, publication in progress).

Since we have already provided evidence that both injury and inflammation are absent in the α7Tg, it is likely that the increased presence of the mesenchymal stem cells will result in the rapid stimulation of myogenesis following exercise. As indicated earlier, progression of myogenesis may occur directly by the entrance of these cells into the myogenic pathway or indirectly by the activation of the endogenous satellite stem cell population.

F. Literature Review Summary

Enhanced α7 integrin provides stabilization necessary to preserve skeletal muscle structure and function and protect against injury. Furthermore, α7Tg mice have an enhanced capability to sequester Sca-1+ CD45- mesenchymal side population cells to areas undergoing great amounts of force during a rigorous exercise protocol. Protection from injury combined with an elevated presence of mesenchymal stem cells would infer a higher degree of myogenesis in the α7Tg animals therefore leading to a preservation of muscular strength following exercise. However, integrin overexpression leading to enhanced myogenesis (and subsequently preserved function) following exercise has never been shown.

Possible implications from such research include contributions to therapies for myopathies such as sarcopenia, muscular dystrophy, and cachexia. Current research in the field is focused on revealing the precise mechanism by which the α7 integrin prevents and repairs damage so that we may fully recognize its therapeutic potential in skeletal muscle.

CHAPTER III.

METHODOLOGY

A single bout of predominantly eccentric or muscle lengthening exercise results in skeletal muscle damage and the initiation of regeneration. In this study, we used forced downhill treadmill running to invoke damage and regeneration in wild type mice and compare the rate and extent of myogenesis between wild type (WT) and α7 transgenic (α7Tg) mice. Following the exercise bout, mice were euthanized at different time points and the gastrocnemius-soleus complex was dissected for histological analyses.

A. Animals

1. α7β1 Integrin Transgenic Mice

Transgenic mice were produced in a SJ6/C57BL6 background at the University of Illinois Transgenic Animal Facility as previously described (Burkin et al., 2001, Boppart et al., 2006). α7Tg mice used for this study express eight-fold higher levels of α7 integrin protein compared to WT mice (Boppart et al., 2006). Genotypes were determined through PCR analysis and α7Tg mice were matched with WT litter mate controls whenever possible.

2. Experimental Design

The protocols for animal use were approved by Institutional Animal Care and Use Committee of the University of Illinois at Urbana-Champaign. The animals were fed *ad libitum* with standard Chow-mixed food and the experiments were conducted at approximately the same time each day. Five week-old female SJL/BL6 WT and α7Tg mice remained at rest (basal) or

were run downhill on a mouse treadmill (Exer-3/6M, Columbus Instruments) (FIGURE 14) at a decline of 20º for 60 min at 17 m/min incorporating a 7-min progressive warm-up beginning at 10 m/min. WT and α7Tg mice randomly assigned to exercise and basal (no exercise) groups were euthanized 2, 4, and 7 days post-exercise (PE) (n ≥ 6/group) (TABLE 1). WT and α7Tg basal controls were treated identically to those in the exercise group and were euthanized at 5 weeks and 4 days of age.

Group	Exercise Status	2D	4D	7D
WT	**B**		**X**	
WT	**EX**	**X**	**X**	**X**
TG	**B**		**X**	
TG	**EX**	**X**	**X**	**X**

TABLE 1. Time points that tissue samples were collected following the single exercise bout (WT = Wild Type Mice: TG = Transgenic Mice; B = Basal; EX = Exercised).

FIGURE 14. Mice were run on a downhill treadmill (Exer-3/6M, Columbus Instruments) set at -20º at 17 m/min for 60 minutes to create a damaging eccentric exercise bout.

B. Histology

1. Tissue Collection

Mice were euthanized via CO_2 gas inhalation in the basal state or 2, 4, and 7 days PE with $\geq$ 6 WT and 6 α7Tg animals assigned to each group. The gastrocnemius-soleus complexes were rapidly dissected (FIGURE 15), flash-frozen in liquid nitrogen-cooled isopentane, and stored at -80°C.

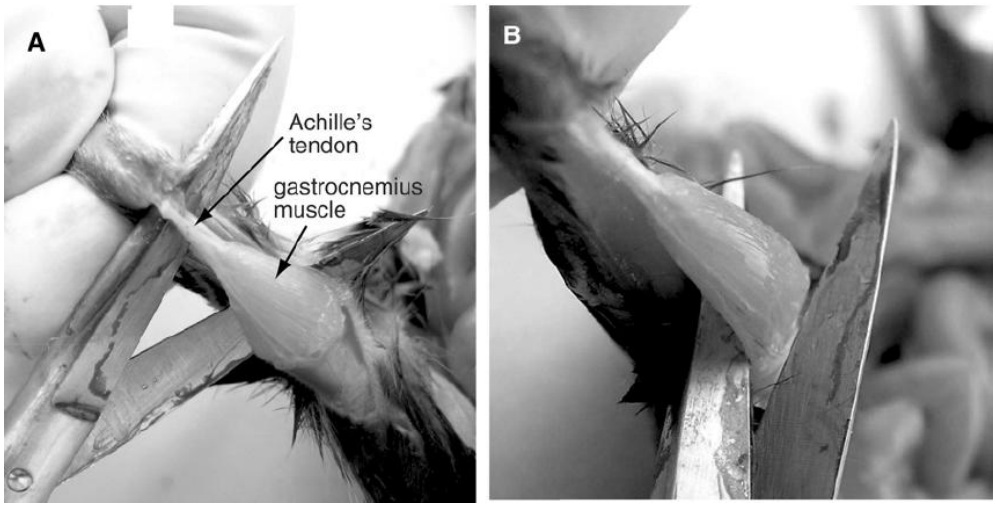


FIGURE 15. Gastrocnemius-Soleus complexes were separated from the bone, the Achille's tendon was severed, and then the proximal end of muscle was transected at the point of origin (Antal et al., 2007).

2. Cryosectioning

Frozen gastocnemius-soleus muscles were cut in half along the axial plane, embedded in OCT (Tissue-Tek; Fisher Scientific, Hanover Park, IL), and 10-µm cryosections were cut (3 sections per sample, separated by 100 µm) distally using a CM1850 cryostat (Leica, Wezlar, Germany). Sections were placed on frozen microscope slides (Superfrost; Fisher Scientific, Hanover Park, IL).

3. Evans Blue Dye

WT and α7Tg mice were injected with Evans blue dye (50 µl/10 g body wt ip) 90 min prior to downhill running. Twenty-four hours PE, gastrocnemius-soleus complexes were dissected as described above. Frozen 10-um-thick sections were prepared (3 sections per sample) and the myofiber damage in each animal was observed qualitatively with the use of fluorescence microscopy (40X objective; Zeiss Axiovert 200M Fluorescent Microscope) to verify that the transgenic animals are protected from injury in the 60 min 17 m/min eccentric exercise.

4. H&E

In order to assess centrally located nuclei (CLN), a measure of regeneration, tissue sections were stained with hematoxylin & eosin (H&E) by an automated robotic slide stainer (Leica Autostainer XL, Leica Instrument, Nussloch, Germany). CLN in regenerating muscles were counted at 20X magnification by brightfield microscopy and researchers were blinded to the treatment (Zeiss Axiovert 200M Microscope). A total of 1000 fibers were observed per animal and numbers of CLN fibers per 1000 fibers are given, averaged per group.

5. eMHC/Sca-1

The transition from myoblast proliferation to terminal differentiation and formation of a new fiber is dependent upon expression of embryonic myosin heavy chain (eMHC) (Carosio et al., 2009). Therefore, myogenesis was detected using eMHC to identify newly regenerating muscle fibers. Slides were fixed in acetone for 5 minutes and blocked with PBS containing 10% horse serum (HS). Endogenous mouse immunoglobulin was blocked with goat anti-mouse monovalent Fab fragments (AffiniPure Fab Fragment Goat Anti-Mouse IgG (H+L), Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) at a 1:20 dilution in 10% HS. Sca-1, the stem cell antigen, is a cell surface glycoprotein expressed on a variety of cell types, including

non-satellite stem cell populations within the skeletal muscle. eMHC and Sca-1 were co-stained in order to assess the proximity of new muscle fiber development to Sca-1 + dense areas of the muscle. eMHC positive fibers were detected with the use of the 47A mouse monoclonal antibody (1:10, kindly provided by the Kaufman Laboratory) and FITC-labeled donkey anti-mouse secondary antibody (1:100, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Sca-1 was detected using the purified Ly-6A/E polyclonal rat anti-mouse antibody (D7 Rat IgG_{2a} κ Ms 1:100, BD bioscience San Jose, CA) and rhodamine-labeled donkey anti-rat secondary antibody (TRITC 1:100, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). The total number of eMHC positive fibers was assessed in 40 fields of view at 20X. Researchers were blinded to the treatment samples.

6. α7 Integrin/Sca-1

Sca-1+ cell density and localization was examined in the days following exercise by co-staining all samples for the α7 integrin and Sca-1. Slides were fixed in acetone for 5 minutes and blocked with PBS containing 5% BSA. Endogenous mouse immunoglobulin was blocked with goat anti-mouse monovalent Fab fragments (AffiniPure Fab Fragment Goat Anti-Mouse IgG (H+L), Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) at a 1:20 dilution in 5% BSA. The rat α7B integrin cytoplasmic domain was localized with the use of purified α7CDB polyclonal rabbit anti-rat antibody (CDB347 1:500, kindly provided by the Kaufman Laboratory) and FITC-labeled donkey anti-rabbit secondary antibody (1:100, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Sca-1 was detected using the procedures listed above.

Fiber cross-sectional areas were measured using the advanced measurements component of Axiovision software (Zeiss) on images of slides stained for the α7 integrin taken at 20X. The

cross-sectional areas of 1,000 fibers from gastrocnemius-soleus muscle of WT and α7Tg, basal and exercised mice (7 days PE) were measured. Areas were averaged per 1,000 fibers and averaged per group.

7. Pax 7

Slides were fixed in acetone for 5 minutes and blocked with 1X PBS containing 10% Horse Serum. Endogenous mouse immunoglobulin was blocked with goat anti-mouse monovalent Fab fragments (AffiniPure Fab Fragment Goat Anti-Mouse IgG (H+L), Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) at a 1:20 dilution in 10% Horse Serum. Both quiescent and activated satellite cells were localized with the use of the purified Pax7 monoclonal mouse antibody (1:200, Developmental Studies Hybridoma Bank at the University of Iowa, Iowa City, Iowa) and FITC-labeled donkey anti-mouse secondary antibody (1:100, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Pax7 positive cells were counted in 40 fields of view per sample and averaged per group.

8. Pax7/Injected SP Cells

In a previous experiment, we had extracted SP cells from α7Tg mice (cell sorted through flow cytometry for Sca-1+ CD45-), labeled them with lipophilic dye 1 (DiI), and injected $1x10^6$ cells i.m. into basal and exercised WT mice (run at -20° at 17 m/min for 30 min). In this experiment, we examined Pax7+ nuclei (using the previously described protocol) in these samples to evaluate the effect of SP cells and exercise on satellite cell activation.

9. Analysis

All immunofluorescence slides were mounted using Vectashield mounting medium containing DAPI (Vector Laboratories, Fisher Scientific, Hanover Park, IL) to identify nuclei,

and sealed with nail polish. Antibodies were detected by fluorescent microscopy (20X and 40X objectives and Excitation570/Emission640 filters; Zeiss Axiovert 200M Fluorescent Microscope) Images were acquired using an AxioCam digital camera (Zeiss) and AxioVision 40V4.8.1.0 software (Zeiss).

C. Functional Testing

Using a separate set of 5-week-old mice, functional testing was assessed to detect any differences in muscle strength between α7Tg and WT mice as well as any strength differences between basal and exercised states. Muscle contractile force of the gastrocnemius (FIGURE 16) was measured *in vivo* for each of the following groups: WT Basal, α7Tg Basal, WT 7D PE, α7Tg 7D PE. On day 7, maximum isometric contraction strength was assessed in hindlimb plantar flexors. The force-measurement apparatus consists of a servomotor and analog control unit (model 305C-LR, Aurora Scientific, Aurora, ON), a square-wave stimulator (model 2100, A-M Systems, Carlsborg, WA), and a PC running a customized LabView 8.2 program to control both the servomotor and the stimulator. The servomotor system was calibrated by placing a series of known weights at a distance of 20mm from the axis and creating a torque/voltage standard curve.

For force measurement, mice were first weighed, then fully anesthetized by the administration of 90-120mg/kg Ketamine, 9-12mg/kg Xylazine IP. Surgical-plane anesthesia was confirmed by pinching the pad of the hind foot. Once the anesthesia took full affect, the mice were placed on a table warmed to 37°C, where all further surgical procedures were performed.

An incision was first made on the lateral thigh of the mouse, parallel and superficial to the femur. At this point the illiotibial band is visible, and the hamstring and quadriceps muscle

groups were dissected apart, allowing access to the sciatic nerve, which sits between the hamstrings and femur. Further dissection of the popliteal fascia allowed the split of the sciatic into the tibial and common peroneal nerves to be visible. The common peroneal branch was identified visually and confirmed by a dorsiflexion muscle twitch upon gentle pinching. At this point the common peroneal nerve was severed, ensuring stimulation of only plantarflexor muscles upon stimulation of the sciatic. The ankle of the mouse was then fixed in the servomotor's footplate, with the talus (the rotational center of the ankle joint) aligned with the servomotor axis. The mouse was then positioned so as to place the knee joint at 90° and the ankle at 90°, where the knee was immobilized with an alligator-clip style clamp about the femoral condyle. Two hooked-needle electrodes were then placed proximal to the knee on the sciatic nerve, with the two electrodes approximately 1mm apart and contacting only the sciatic and not adjacent muscle tissue. The sciatic nerve was then stimulated at 250 hz for 1.5 seconds to evoke a maximal contraction, during which time the servomotor held the ankle to 90° in order to measure isometric force production. This stimulation was repeated 9 times with a recovery period of 5 seconds between contractions (6.5 seconds from contraction 1's start to contraction 2's start), for a total of 10 measurements. The highest of the 10 acquired data points was taken as the maximum. Data were recorded by a customized LabView program (National Instruments) to provide a plot of ankle-torque/time.

Mice remained in an anesthetized state during the experiment. Following the completion of mechanical measurements, mice were euthanized via cervical dislocation, gastrocnemius-soleus complexes were dissected, weighed, and flash frozen in liquid nitrogen cooled isopentane. The values of power output (grams) were normalized with respect to muscle mass (grams/mg muscle weight).

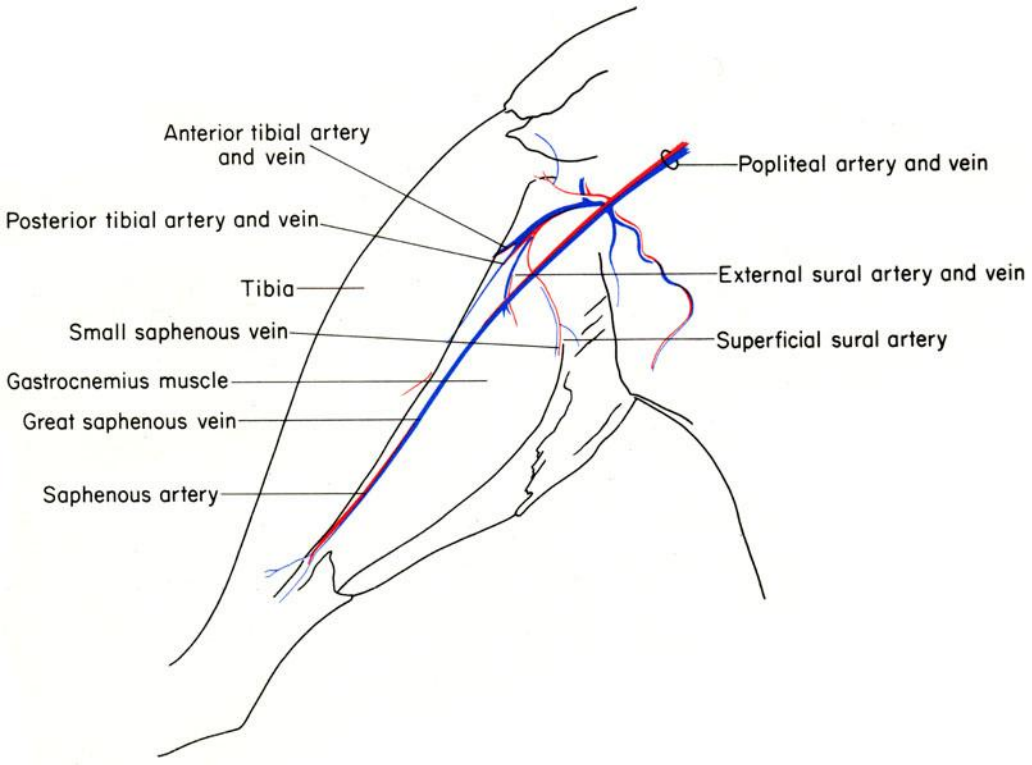


FIGURE 16. Illustration of the anatomy of the mouse hind limb (Cook, 1965).

D. Statistical Analysis

All averaged data are presented as the means ± SE. Comparisons between WT and α7Tg mice, basal and exercised, were performed by two-way ANOVA to determine if an interaction effect was observed for group and time. To determine significance, comparisons between groups were performed by one-way ANOVA, followed by Tukey's post hoc analysis (SigmaStat). Differences were considered significant at $P < 0.05$.

CHAPTER IV.

RESULTS

A. The Overexpression of the α7β1 Integrin is Effective in Increasing Myogenesis

During myogenesis, satellite cells become activated (for example by growth factors secreted by macrophages) and begin to proliferate at about 24 hrs post-injury within the preserved basement membrane (Rantanen, Hurme, Lukka, Heino, & Kalimo, 1995). They differentiate into myoblasts, stop dividing, and fuse with existing mature fibers or with one another to form small-caliber multinucleated myotubes expressing the embryonic form of myosin heavy chain (eMHC). As the *de novo* myotubes gradually mature, they express the adult myosin heavy chain (MHC) and their myonuclei become more peripherally located (Hurme, Kalimo, Lehto, & Jarvinen, 1991).

In this experiment, I verified that a single eccentric exercise bout induced myogenesis in WT mice. Furthermore, I tested if the overexpression of the α7β1 integrin in muscle resulted in enhanced and acclerated myogenesis following exercise. I used a range of markers to evaluate new fiber synthesis, including: the expression of eMHC, the deviation of the nucleus from the periphery to the center of the fiber (CLN), and satellite cell activation (Pax7).

1. eMHC

As shown in FIGURE 17, small caliber, triangular-shaped eMHC+ fibers with overdeveloped nuclei were detected in representative WT and α7Tg mice in the days following a single bout of downhill running exercise compared to the appropriate basal controls. The total

number of eMHC+ fibers appeared to be greater in the α7Tg mice as early as 2-days PE (FIGURE 18), then declined at 7-days PE, when these fibers presumably enlarged and began expressing the mature form of myosin heavy chain. Brightly stained, small caliber eMHC+ fibers were also observed in WT mice beginning at 4-days and were maximal at 7-days PE (FIGURE 18). eMHC staining was never observed in large caliber fibers in WT or α7Tg mice, suggesting that satellite cell fusion with mature fibers did not occur in response to a single bout of eccentric exercise.

The total number of eMHC+ fibers were then counted in a total of 40 fields in both WT and α7Tg mice. As shown in FIGURE 19, a trend toward an increase in eMHC+ fibers was observed in WT mice from basal (7.28 ± 2.68) to 7-days PE (46.17 ± 9.32) which is a standard response to exercise-induced injury. While the eMHC+ fiber levels were comparable between the WT and α7Tg groups at baseline (7.28 ± 2.68 and 12.89 ± 3.43), a marked and maximal 5-fold increase in eMHC+ fibers was observed in α7Tg mice at 2-days (66.94 ± 16.20) ($P<0.001$). The total number of new fibers remained elevated in α7Tg mice at 4-days PE (58.34 ± 18.32) ($P<0.001$), and then gradually declined at 7-days PE (49.27 ± 16.27). These results demonstrate that a single bout of eccentric exercise stimulates myogenesis in mice and that the overexpression of the α7β1 integrin accelerates this process.

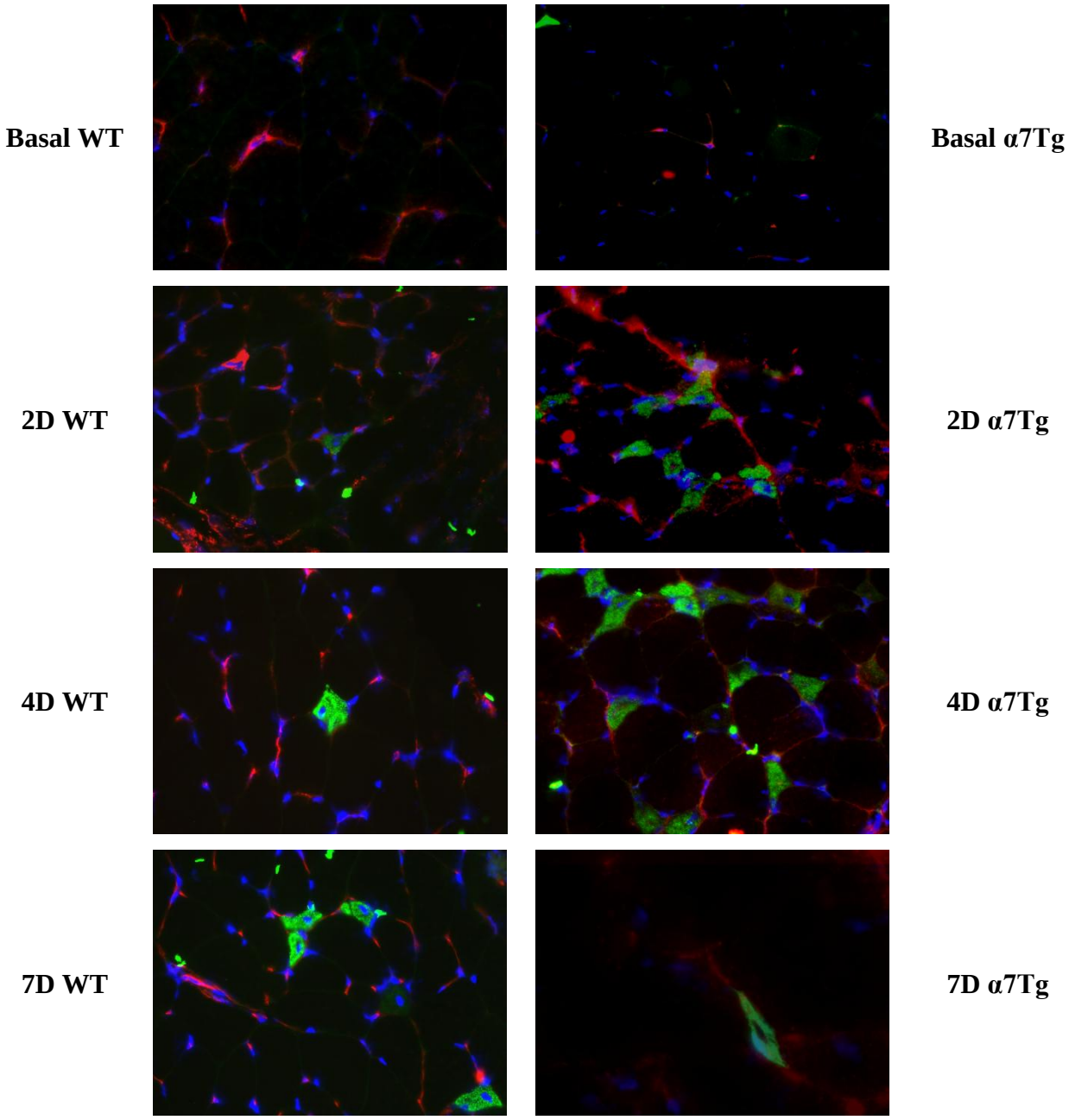


FIGURE 17: Embryonic myosin heavy chain stain for eMHC positive fibers (green) with centrally located nuclei (blue) and surrounding Sca-1+ cells (red) in WT and α7Tg mice following a single bout of eccentric exercise.

A. 2D α7Tg

B. 7D WT

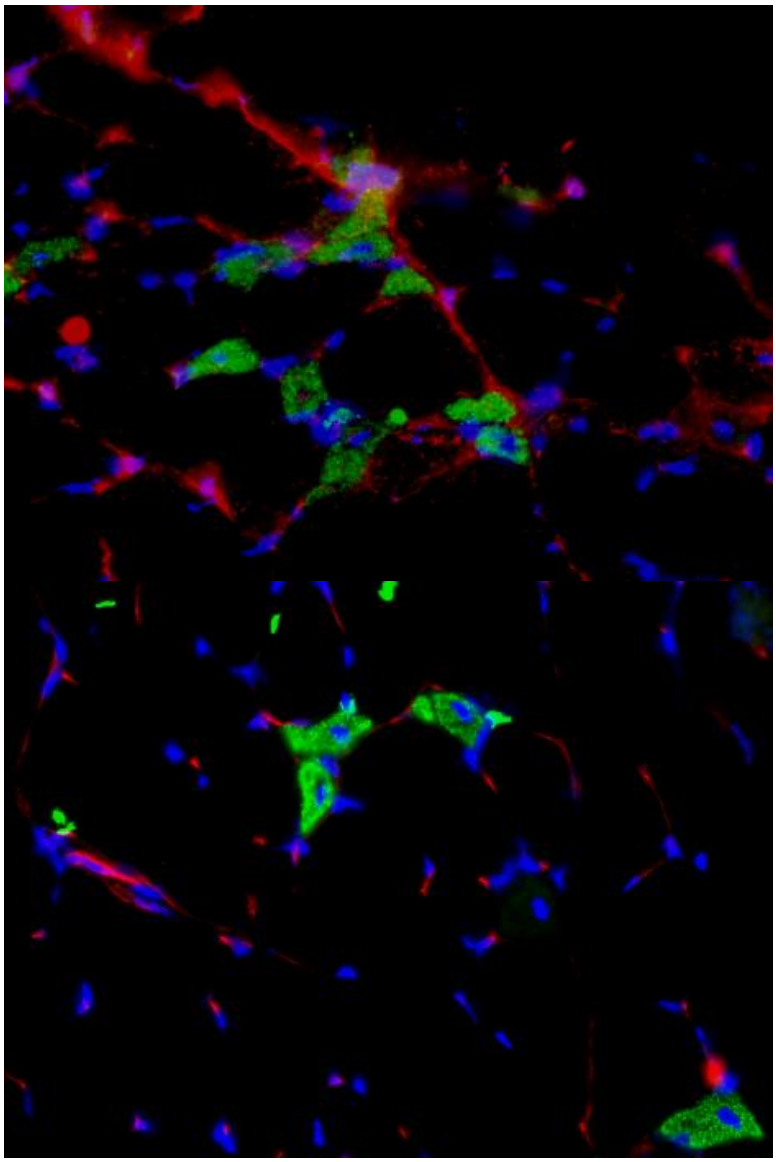


FIGURE 18: Enlargement of select images from FIGURE 17. **A.** eMHC staining in an α7Tg mouse at 2-days PE and **B.** eMHC staining in a WT mouse at 7-days PE enlarged to illustrate eMHC positive fibers (green) with centrally located nuclei (blue) with surrounding Sca-1+ cells (red).

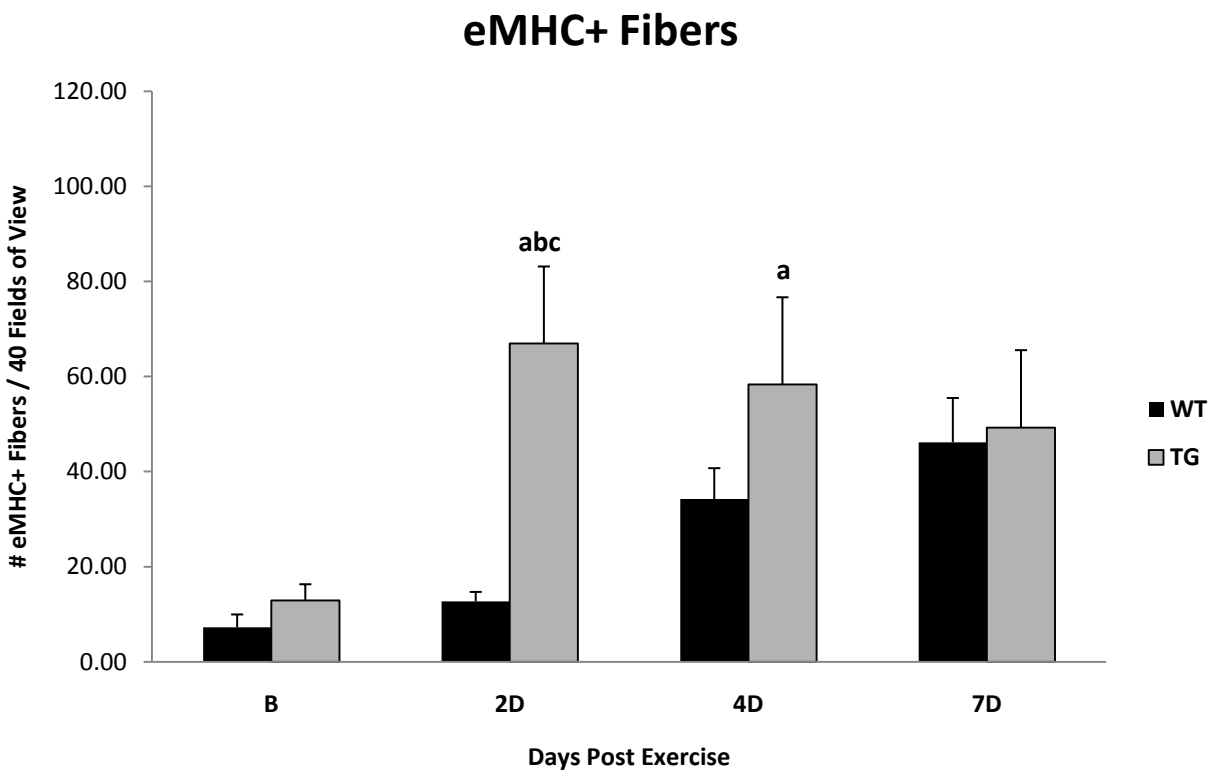


FIGURE 19: Myogenesis is accelerated in α7Tg mice. WT and α7Tg mice remained at rest or were run downhill (20° decline) at 17 m/min for 60 min (n = 6-9/group). Gastrocnemius-soleus complexes were dissected at 2, 4 and 7-days PE and cryosections were examined for eMHC expression. Values are means ± SE. **a** = 2D α7Tg and 4D α7Tg significantly different from basal WT (P < 0.001), **b** = 2D α7Tg significantly different from basal α7Tg (P<0.001), **c** = 2D α7Tg significantly different from 2D WT (P<0.001).

	n	# eMHC+ Fibers	SE
B WT	6	7.28	± 2.68
B TG	9	12.89	± 3.43
2D WT	6	12.72	± 1.98
2D TG	5	66.94[abc]	± 16.20
4D WT	6	34.22	± 6.51
4D TG	5	58.34[a]	± 18.32
7D WT	6	46.17	± 9.32
7D TG	7	49.27	± 16.27

TABLE 2: eMHC stain for regenerating fibers in 40 fields of view (averaged over 3 counted sections per sample).
[a] = 2D α7Tg and 4D α7Tg significantly different from basal WT (P<0.001)
[b] = 2D α7Tg significantly different from basal α7Tg (P<0.001)
[c] = 2D α7Tg significantly different from 2D WT (P<0.001)

2. CLN

During myogenesis, satellite cells appear to increase in size and deviate from their usual peripheral location on the fiber migrating towards the center of the fiber. This position allows for efficient assembly of newly translated myofibrillar proteins into centrally located sarcomeres. Therefore, identification of centrally located nuclei (CLN) in the fiber provides an alternative method for evaluation of myogenesis (Carosio et al., 2009).

Examination of eMHC stained samples showed the consistent presence of nuclei in the central position as identified by DAPI (blue) immunofluoresence (FIGURE 17). However, mature fibers also displayed CLN in the days following exercise, characteristic of fibers that have completed the process of myogenesis. Samples were stained with hematoxylin and eosin to obtain a clear and stable image of CLN within all fibers, thus providing an extended view of the myogenic process. The rate of appearance of CLN was similar to the results obtained with eMHC staining (FIGURES 20 & 21). As shown in FIGURE 20, a gradual trend toward an increase in CLN was observed in WT mice in the days following exercise, with maximal numbers observed at 7-days PE. In the α7Tg mice, a significant 5-fold increase in CLN was observed at 2-days PE (77.83 ± 13.04) compared to the α7Tg basal control (14.56 ± 1.85) ($P < 0.001$). By two-way ANOVA, an interaction effect was observed between group and time.Whereas most of the CLN+ fibers were small caliber at 2-days PE, most were in larger fibers by 7-days PE. Thus, these results are in accord with the eMHC data and suggest that newly generated fibers are sustained at 7-days PE. In addition, the drop in CLN in the α7Tg mice at 4- and 7-days PE may reflect fusion of eMHC+ fibers, resulting in the generation of large fibers.

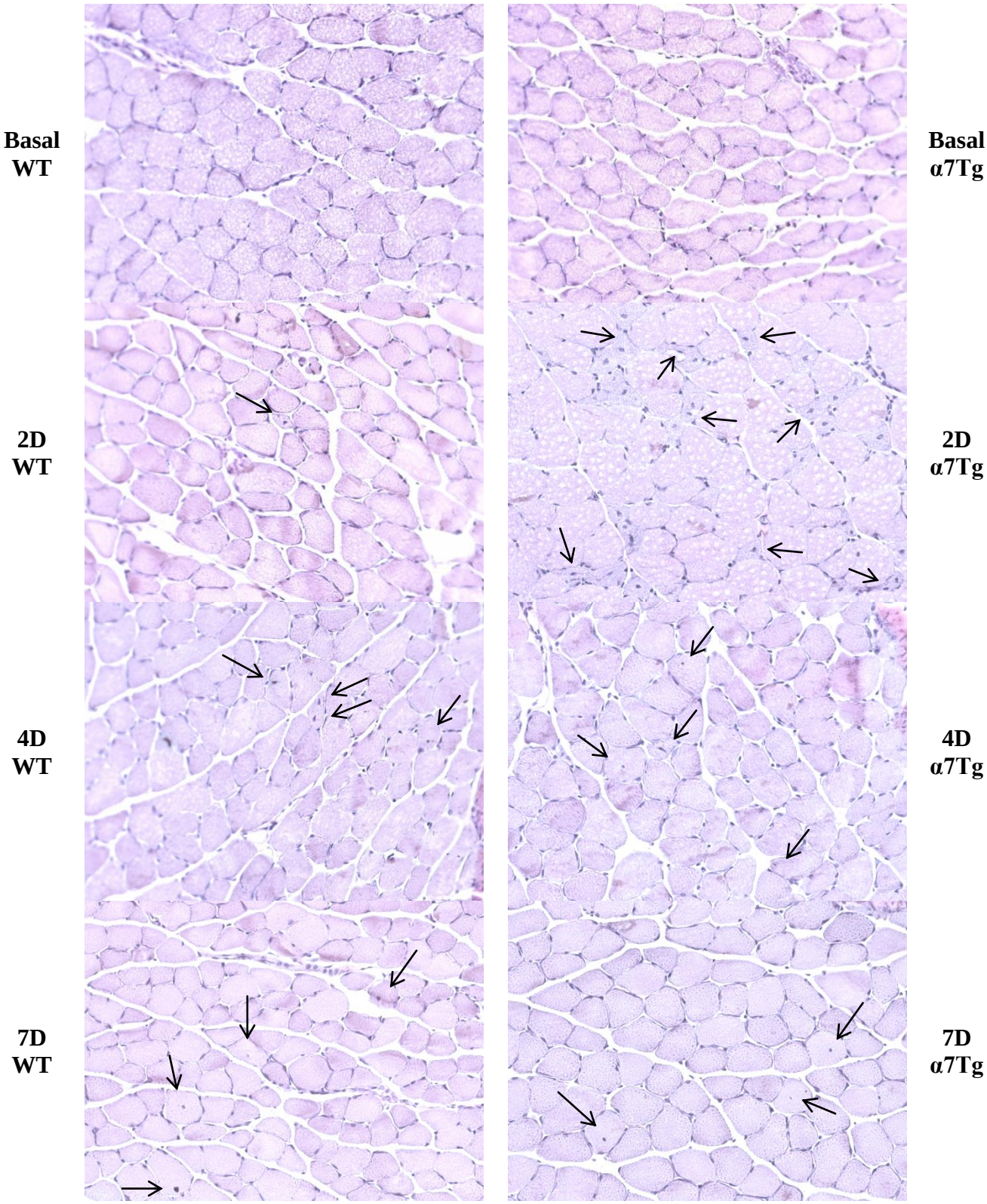


FIGURE 20: H&E stain for CLN (purple) in WT and α7Tg mice following a single bout of eccentric exercise.

A. 2D α7Tg

B. 7D α7Tg

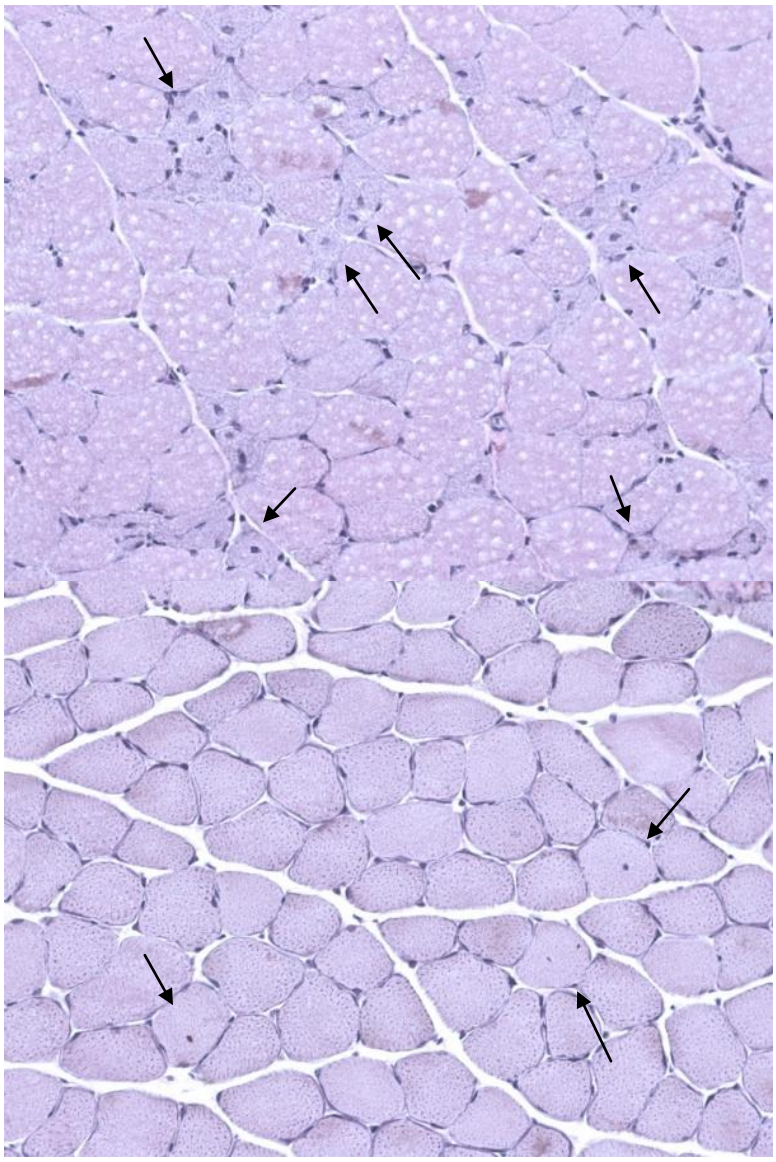


FIGURE 21: Enlargement of select images from FIGURE 20. **A.** H&E staining in an α7Tg mouse 2-days PE and **B.** H&E staining in an α7Tg mouse 7-days PE enlarged to illustrate CLN in small vs. large caliber fibers.

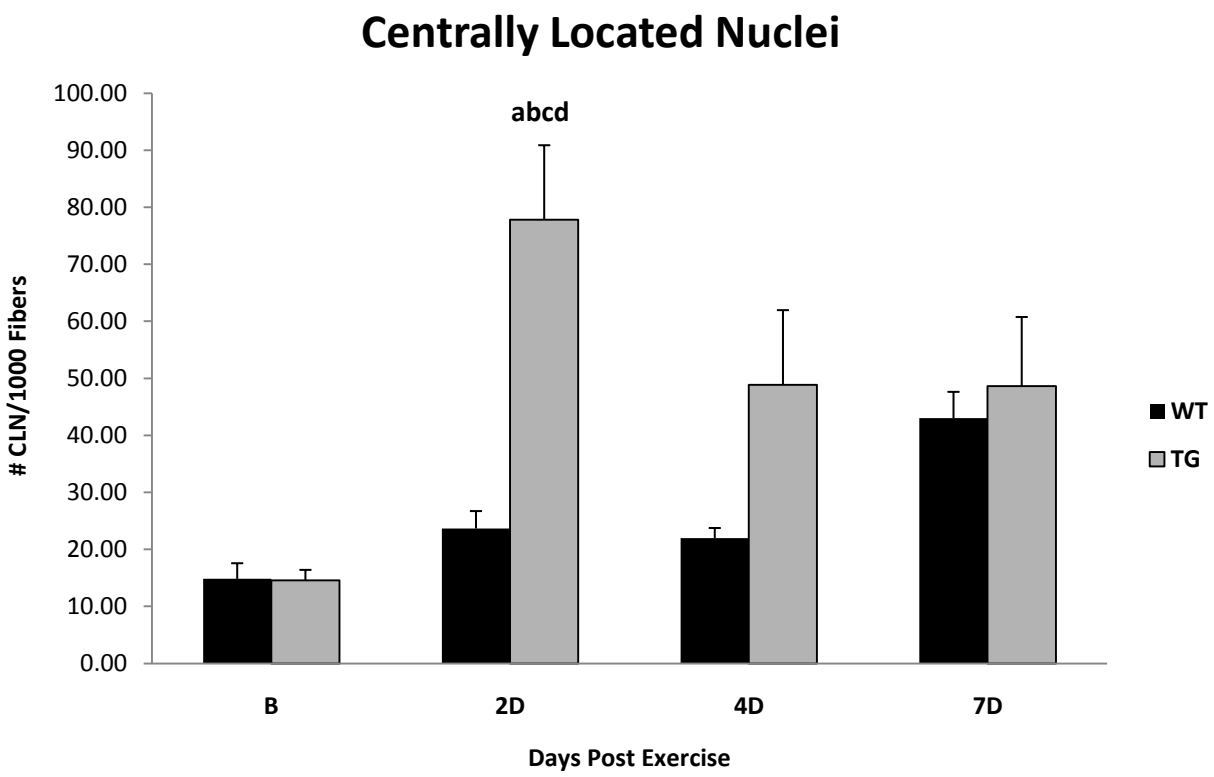


FIGURE 22: α7Tg mice have elevated levels of CLN PE. WT and α7Tg mice remained at rest or were run downhill (20° decline) at 17 m/min for 60 min (n = 6-9/group). Gastrocnemius-soleus complexes were dissected at 2, 4 and 7-days PE and cryosections were examined for CLN. Values are means ± SE. **a** = 2D α7Tg significantly different from basal WT ($P < 0.001$), **b** = 2D α7Tg significantly different from basal α7Tg ($P<0.001$), **c** = 2D α7Tg significantly different from 2D WT ($P<0.001$), **d** = 2D α7Tg significantly different from 4D WT ($P<0.001$).

	n	Average # CLN	SE
B WT	6	14.83	± 2.74
B TG	9	14.56	± 1.85
2D WT	6	23.67	± 3.05
2D TG	6	77.83[abcd]	± 13.04
4D WT	6	22.00	± 1.75
4D TG	6	48.83	± 13.12
7D WT	6	43.00	± 4.63
7D TG	8	48.63	± 12.13

TABLE 3: H&E stain for CLN (counted in 1,000 fibers).
[a] = 2D α7Tg significantly different from basal WT ($P<0.001$)
[b] = 2D α7Tg significantly different from basal α7Tg ($P<0.001$)
[c] = 2D α7Tg significantly different from 2D WT ($P<0.001$)
[d] = 2D α7Tg significantly different from 4D WT ($P<0.001$)

C. Sca-1+ CD45- Stem Cells Indirectly Promote Myogenesis

1. Evaluation of the Direct Contribution of Sca-1+CD45- Cells to Myogenesis

Mesenchymal-like Sca-1+ CD45- stem cells are upregulated in several areas of skeletal muscle in α7Tg mice (Boppart et al., publication in progress). These cells have previously been shown to directly and indirectly contribute to myogenesis *in vitro* and *in vivo* in response to chemical-induced injury (Motohashi et al., 2008; Uezumi et al., 2006). However, it is not known whether these stem cells contribute to the process of myogenesis following eccentric exercise.

eMHC+ fibers were first examined in Sca-1+ cell dense areas of the muscle, including the interstitium, vessel-nerve bundles, and the myotendinous junction. Although eMHC staining was never observed in these areas of the muscle, small caliber fibers containing peripheral nuclei were consistently detected in the interstitium between the gastrocnemius and soleus muscles (FIGURE 23). These cells were confirmed to be muscle fibers based on fiber type immunohistochemistry (FIGURE 23C). It is possible Sca-1+ cells contributed to the formation of these small caliber fibers, but this cannot be determined without tracking the cells with a marker that is retained during the differentiation process.

A. H&E

B. α7/Sca-1 40X

C. Type I & II Fibers

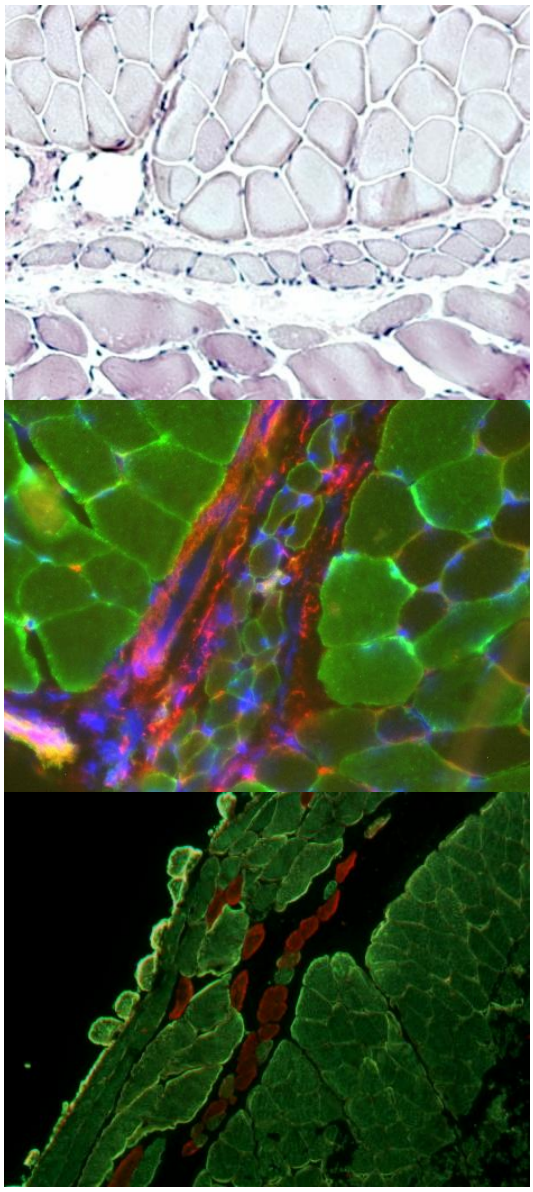


FIGURE 23. Fibers Found in the Interstitium Identified by H&E, α7/Sca-1, and Fiber Type Stains. **A.** H&E staining of the small fibers reveals no CLN. **B.** α7β1 integrin labeled with FITC (green), Sca-1+ cells labeled with TRITC (red), and nuclei labeled with Dapi (blue). **C.** Fiber Type Staining: Type I fibers labeled with FITC (green) and type II fibers labeled with TRITC (red).

In a previous experiment performed in our laboratory, Sca-1+ CD45- stem cells were isolated from α7Tg skeletal muscle, labeled with a dye (lipophilic DiI) that fluoresces red, and injected into one leg of two immunodeficient WT mice 1 hour after remaining at rest or completing a 30 min bout of eccentric treadmill exercise The contralateral leg was injected with

saline as a control. Muscles were then dissected 48 hr post exercise. To determine the direct contribution of these cells to myogenesis, eMHC expression was evaluated in DiI+ cells. Co-expression of DiI with eMHC was rarely observed (data not shown). One limitation of this experiment is that the Sca-1+CD45- cells were never exposed to strain and this mechanical event may be necessary to induce myogenesis. This question is currently being addressed in the laboratory.

2. Evaluation of the Indirect Contribution of Sca-1+CD45- Cells to Myogenesis

Pax7 is a satellite cell-specific marker that is expressed in both quiescent and activated cells, yet upregulated in response to myogenic stimuli, such as growth factors. Samples injected with DiI labeled Sca-1+CD45- stem cells were evaluated for Pax7 expression to determine whether the presence of Sca-1+CD45- stem cells stimulates endogenous satellite stem cell activation (FIGURE 24).

The level of Pax7+ cells in the basal samples were comparable to one another (basal saline: 63.33, basal stem cells: 71.20), whereas the number of cells in the exercised saline leg was lower (47.75). Interestingly, the exercised stem cell-injected leg had drastically increased levels of Pax7+ cells (111.25) (FIGURE 25). The activated satellite cells were not in proximity to the injected cells, making it unlikely that injected Sca-1+CD45- stem cells were upregulating Pax7 and entering the satellite stem cell niche.

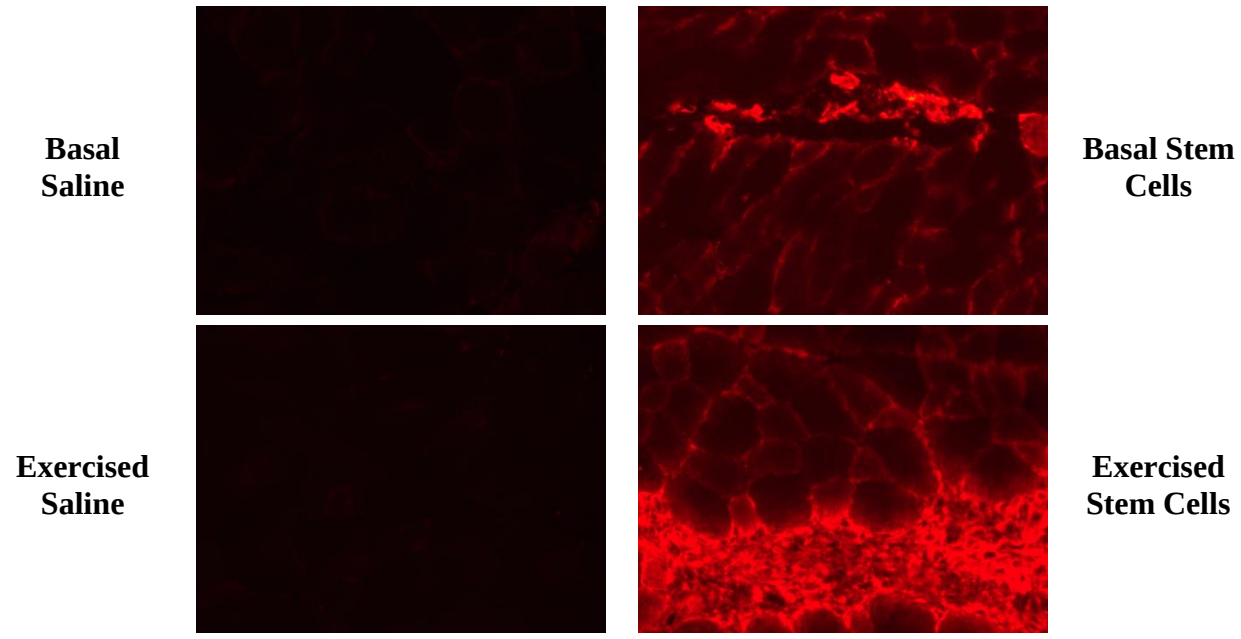


FIGURE 24: Presence of injected Sca-1+ CD45- cells labeled with DiI in exercised WT animals.

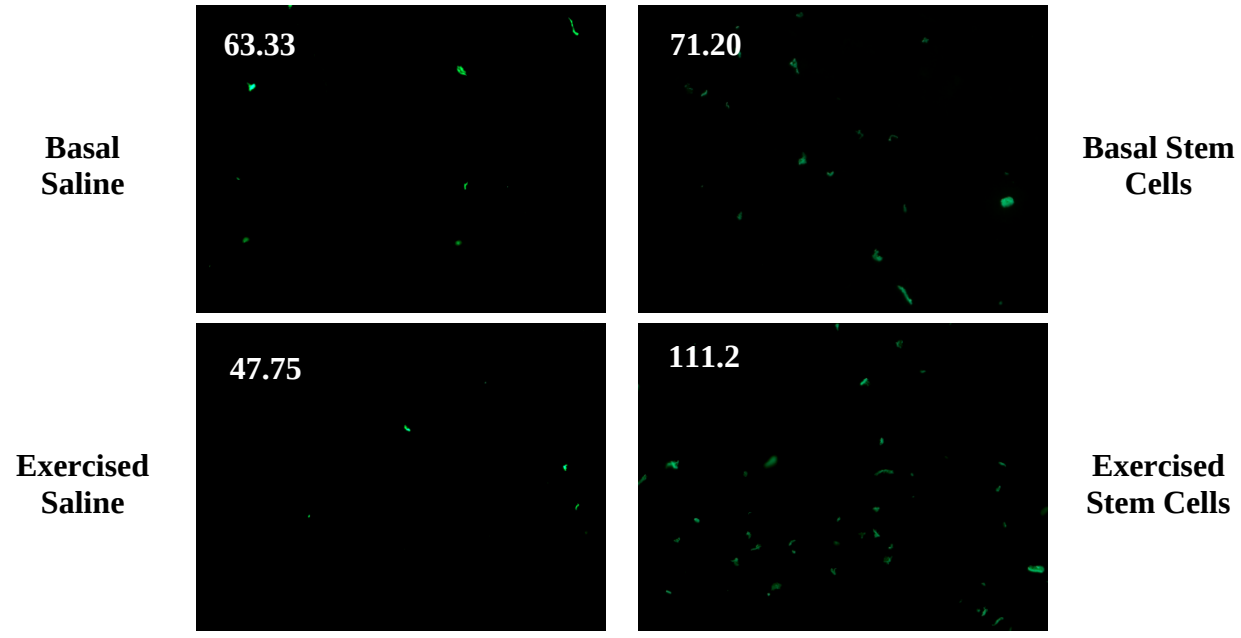


FIGURE 25: Pax7 stain indicating satellite cell activation in exercised WT animals injected with Sca-1+ CD45- cells in 40 fields of view (average number of Pax7+ cells per field of view denoted in top left corner of image).

The results of these preliminary experiments provide evidence that satellite cells are likely activated to a greater extent in α7Tg mice compared to WT mice and may be responsible for the accelerated myogenesis observed. As shown in FIGURE 26, Pax7 expression is upregulated 2-days PE in WT mice (57.89 ± 5.80) ($P<0.01$) which gradually decreased as the time points progressed. This response is characteristic of normal, WT mice as the satellite cells become activated following the exercise-induced injury and Pax7 expression begins declining as the myogenic process progresses (Carosio et al., 2009). Pax7 expression was elevated in the α7Tg compared to WT mice in the basal state (50.34 ± 8.26) ($P < 0.01$) and did not increase to a further extent at 2-days PE (52.88 ± 5.45) ($P<0.01$).

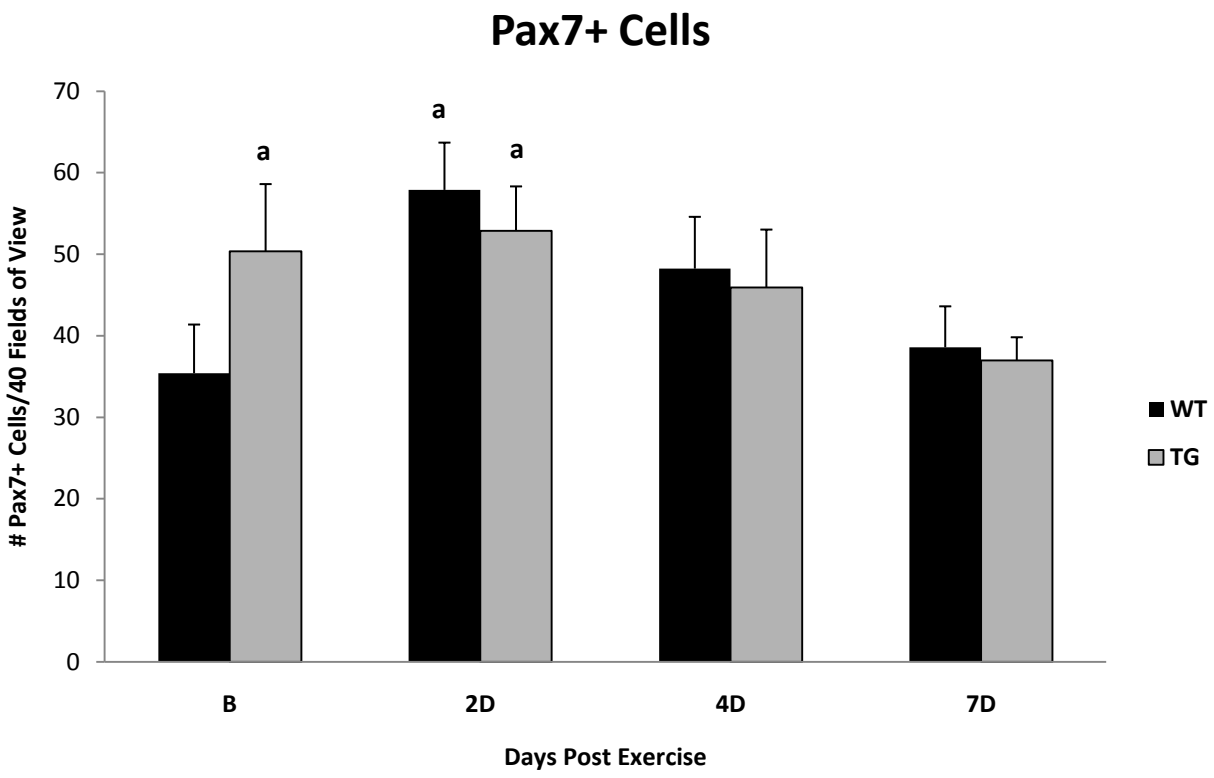


FIGURE 26: α7Tg mice have elevated baseline levels of Pax7+ cells and both α7Tg and WT mice increased in Pax7+ fibers at 2-days PE. WT and α7Tg mice remained at rest or were run downhill (20° decline) at 17 m/min for 60 min (n = 4/group). Gastrocnemius-soleus complexes were dissected at 2, 4 and 7-days PE and cryosections were examined for Pax7. Values are means ± SE. **a =** basal α7Tg, 2D α7Tg, and 2D WT significantly different from basal WT ($P < 0.01$).

	n	Average # Pax7	SE
B WT	4	35.40	± 5.98
B TG	4	50.34[a]	± 8.26
2D WT	4	57.89[a]	± 5.80
2D TG	4	52.88[a]	± 5.45
4D WT	4	48.23	± 6.36
4D TG	4	45.94	± 7.09
7D WT	4	38.58	± 5.03
7D TG	4	36.96	± 2.85

TABLE 4: Pax7 stain for satellite cell activation (in 40 fields of view).
[a] = basal α7Tg, 2D α7Tg, and 2D WT significantly different from basal WT ($P < 0.01$)

D. Transgenic Mice are Protected from Functional Deficits Post-Injury

An increase in myogenesis without the presence of injury may result in enlargement of the muscle and a subsequent increase in strength. Therefore, muscle force was measured in WT and α7Tg mice 7-days PE. Force output was measured in grams of force and expressed in grams per milligram of muscle weight.

At baseline, WT mice (0.21629 ± 0.01129) had a higher average force output than the α7Tg mice (0.17713 ± 0.00528). At 7-days PE, a trend towards a decrease in force was observed in the WT mice (0.16267 ± 0.02586), reflective of recovery from injury, whereas a trend towards an increase in force was observed in the α7Tg mice (0.24258 ± 0.03275), perhaps due to the presence of new fibers (FIGURE 27).

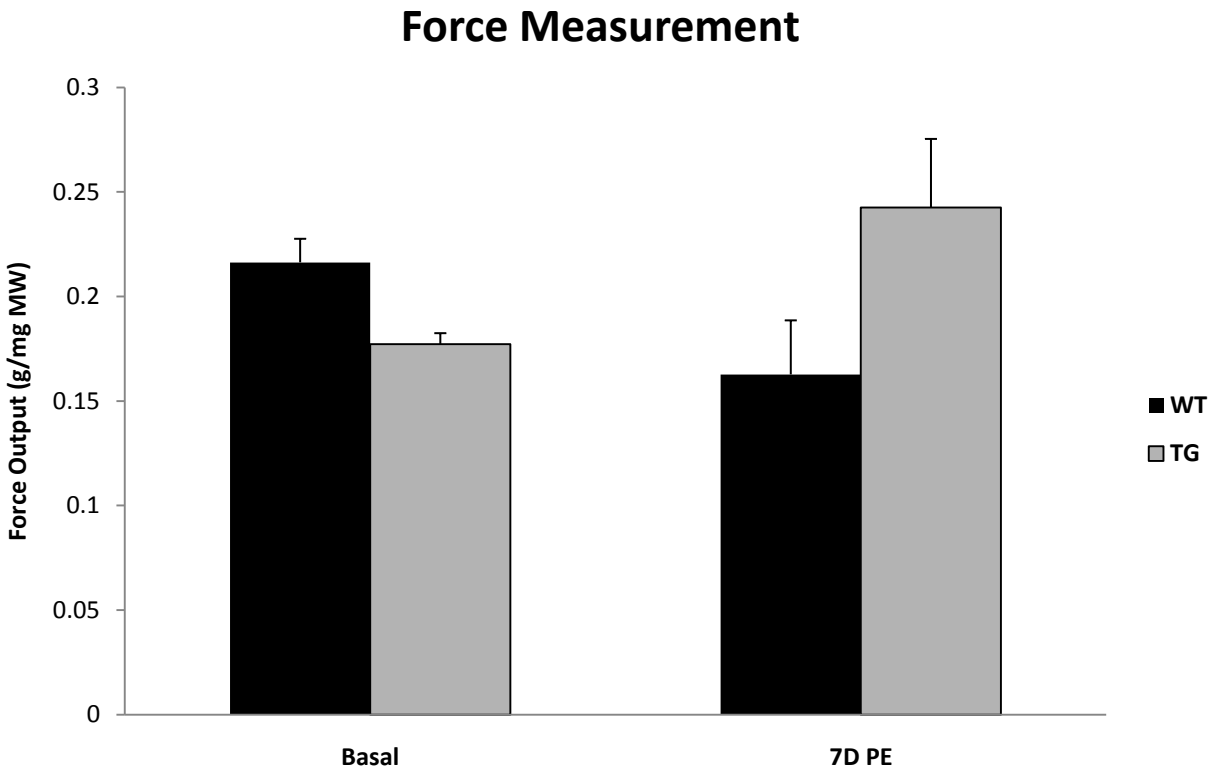


FIGURE 27: At 7-days PE, α7Tg mice are protected from functional deficits and have a trend towards an increased force output while the WT mice have a trend towards a decrease in force output.

	n	Force Output (g/mg)	SE
B WT	4	0.21629	± 0.01129
B TG	4	0.17713	± 0.00528
7D WT	2	0.16267	± 0.02586
7D TG	4	0.24258	± 0.03275

TABLE 5: Isometric force measurement data in WT and α7Tg Mice 7-days PE (expressed in grams of force per milligrams of muscle weight).

E. Transgenic Mice Have Larger Muscle Fibers

To determine if the preservation in force was due to individual muscle fiber hypertrophy or the presence of new fibers in the α7Tg mice, cross sectional areas (CSA) of the muscle fibers were evaluated in 1,000 fibers from a subset of mice in both WT and α7Tg groups in the basal state and at 7-days PE. A trend towards an increase in CSA was observed in both the WT mice from basal (1261.42 ± 301.38) to 7-days PE (1566.744 ± 23.30) and α7Tg mice from basal (1448.79 ± 332.95) to 7-days PE (1735.338 ± 60.54) with the α7Tg mice having a trend towards slightly larger fiber CSAs. Thus, the increase in force is likely due to both increased myogenesis and enlargement of existing or regenerating fibers.

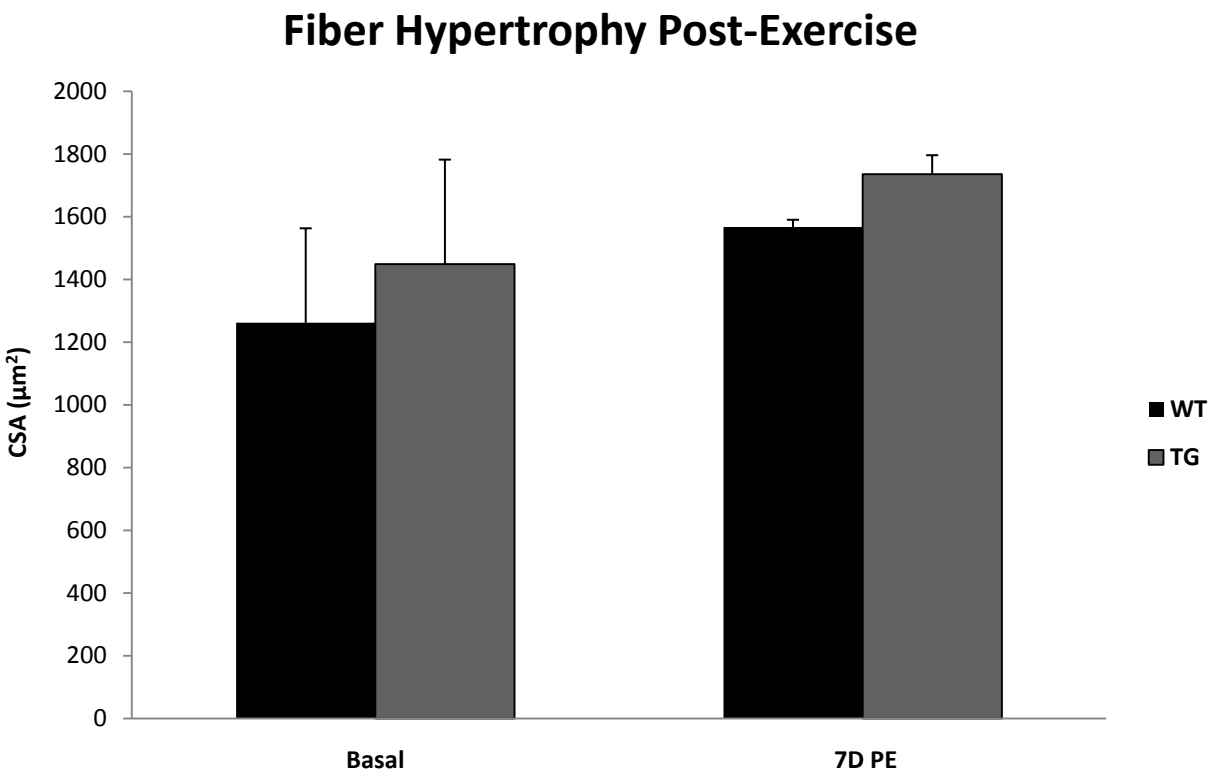


FIGURE 28: α7Tg mice have a trend towards slightly larger fiber CSA than WT mice at baseline and both groups have a trend towards hypertrophy at 7-days PE.

	n	CSA (μm^2)	SE
B WT	2	1261.42	± 301.38
B TG	2	1448.79	± 332.95
7D WT	2	1566.74	± 23.30
7D TG	4	1735.34	± 60.54

TABLE 6: Fiber CSA of WT and α7Tg Mice 7-days PE (areas are averaged over 1,000 counted fibers).

CHAPTER V.

DISCUSSION

A. The Overexpression of the α7β1 Integrin Accelerates Myogenesis

Although many aspects of the myogenic developmental program have been discovered, the mechanisms by which the extracellular matrix and the integrin cell surface receptors participate in skeletal muscle repair after injury are not well understood. $\alpha7^{-/-}$ mice have been shown to exhibit decreased membrane integrity and delayed muscle repair, suggesting that the α7 integrin is required for sufficient muscle repair following injury (Rooney et al., 2009). Boppart et al. (2008) demonstrated that the transgenic mice overexpressing the α7 integrin in skeletal muscle are protected from exercise-induced injury and that this integrin is essential for the migration of mesenchymal-like stem cells (MSC). Therefore, I tested the hypothesis that the overexpression of the α7 integrin in transgenic animals would also confer enhanced and accelerated myogenesis following a single bout of strenuous exercise.

To test whether upregulation of the α7 integrin could improve myogenesis, α7 transgenic (α7Tg) mice were subjected to a damaging exercise protocol and evaluated for embryonic myosin heavy chain (eMHC) expression and the presence of centrally located nuclei (CLN) at 2-, 4-, and 7-days post-exercise (PE). Marked 5-fold increases in eMHC expression and CLN were observed in the transgenic mice at 2-days following the exercise bout, whereas more gradual increases in these parameters were observed in WT mice, with myogenic markers becoming maximal at 7-days (PE). Although it is clear that the rate of myogenesis is significantly accelerated in the α7Tg mice, it is not possible to conclude that myogenesis was enhanced to a greater extent compared to WT mice since levels were still increasing at 7-days PE and further

time points were not examined in this study. eMHC+ fibers increased in size from 2-days to 7-days, demonstrating growth of the newly generated fibers observed 2-days PE in the α7Tg mice. As expected during the maturation process, intensity of the fluorescent signal for eMHC declined in the larger fibers, reflecting transition of expression to the adult myosin heavy chain isoform. The decline in eMHC+ fibers in α7Tg mice at 4-days PE suggests these cells rapidly undergo the developmental process. Verification of new fiber maturation can be obtained from the CLN data since this parameter should be maintained during the 7-day period. In the α7Tg mice, fibers with CLN were predominantly small early on following exercise, whereas fibers with CLN were larger by day 7. However, the total number of CLN+ cells was still lower at 4- and 7-days PE. Although it has never previously been described in the literature, it is possible that the small caliber eMHC+ cells fused with one another and the CLN aligned longitudinally in the newly synthesized fiber. Interestingly, small eMHC+ fibers were found in close proximity to one another at 2-days PE, whereas larger CLN+ fibers were never found in close proximity at 7-days PE.

Another interesting observation made during the course of this study is that eMHC expression and CLN were never found in large caliber fibers in the α7Tg mice at 2-days PE, suggesting that satellite cells preferentially fused to form new fibers versus donating their nuclei to preexisting fibers. This finding contradicts most review articles supporting myoblast fusion as a primary mechanism for regeneration following exercise. Although hyperplasia is most commonly associated with animal studies, satellite cell fusion is actually a rare event (Crameri et al., 2004). However, eMHC+ cells are observed in human skeletal muscle following both endurance and resistance exercise (Kadi et al., 1999).

B. Sca-1+ CD45- Side Population Stem Cells Indirectly Promote Accelerated Myogenesis in α7Tg Mice

Our laboratory has previously demonstrated that MCK-mediated α7 integrin expression increases the presence of Sca-1+ CD45- stem cells in skeletal muscle in the basal state and this effect is amplified 24 hrs following exercise. Using similar muscle-derived Sca-1+CD45- stem cells, Motohashi et al. (2008) have shown that these cells indirectly enhance regeneration following injury by significantly increasing satellite cell migration and transplantation. In this study, injection of the Sca-1+ CD45- stem cells into previously exercised mice increased the activation of the endogenous satellite cell population, as evidenced by upregulation of Pax7 expression in cells distally located from the injection site. Although Sca-1+CD45- cells have the potential to become myogenic, our DiI-labeled Sca-1+CD45- cells did not express eMHC. A more thorough analysis of satellite cell activation in samples collected for this study revealed that α7Tg animals have much higher resting levels of Pax7+ cells compared to WT mice. Whereas Pax7+ cells were elevated at 2-days PE in WT mice, no change was observed in the α7Tg mice. There are two possible explanations for the lack of activation. First, it is possible that the satellite cells in the α7Tg mice are "pre-activated" and ready to differentiate upon initiation of the exercise stimulus. Second, enhanced satellite cell activation may have occurred prior to analysis on day 2. Together, these data suggest that Sca-1+CD45- cells in α7Tg mice indirectly contribute to accelerated myogenesis. However, an extension of our preliminary studies using labeled stem cells is required to definitively describe a role for the cells in myogenesis.

Several reports have demonstrated that MSCs secrete a variety of cytokines and growth factors, which suppress the local immune system, inhibit fibrosis and apoptosis, enhance angiogenesis, and stimulate mitosis and differentiation of tissue-specific stem cells (Arnold et al.,

2007; Cassano et al., 2009; Motohashi et al., 2008). Sca-1+ CD45- stem cells extracted from α7Tg mice following exercise secrete LIF, a factor that has been shown to increase the activation of satellite cells (Boppart, unpublished data). It will be interesting to determine whether LIF or other muscle growth-promoting factors are released from Sca-1+ CD45- cells and whether the rate of release increases upon exercise or isolated cellular strain *in vitro*.

Sca-1+ CD45- cells do have myogenic potential (Asakura et al., 2002; Gussoni et al., 1999). Despite the fact that the DiI-labeled Sca-1+ CD45- stem cells in the preliminary study did not express eMHC, it is possible that exposure to strain is necessary for their conversion. In addition, it is also possible that the stem cells were not given enough time to complete the conversion. Evaluation of eMHC occurred in the preliminary study at 2-days PE, whereas higher numbers of eMHC+ cells are not observed in WT mice until 7-days PE. A more thorough analysis of Sca-1+ CD45- stem cell myogenic potential in muscle following exercise and isolated strain *in vitro* is necessary before we can decisively conclude that these cells are solely providing an indirect role in exercise-induced myogenesis.

Although the presence of Sca-1+ CD45- stem cells may be responsible for accelerated myogenesis in α7Tg mice, it is also possible that the α7 integrin simply protects muscle from damage and inflammation, factors which normally delay the onset of myogenesis. In addition, exercise may facilitate α7 integrin-mediated changes in extracellular matrix (ECM) gene expression, including ECM components known to enhance satellite and non-satellite stem cell activation and differentiation. In a study by Ambrosio et al. (2010) the engraftment of injected muscle-derived stem cells was greatly improved following exercise training presumably due to suppression of fibrosis and the presence of an environment more conducive to myogenesis. Specifically, several studies have suggested that laminin promotes myoblast survival,

proliferation, migration, and differentiation (Rooney et al., 2009). It will be a challenge to dissect the precise mechanisms by which the α7 integrin can enhance myogenesis and *in vitro* studies undoubtedly will be necessary to examine the possibilities suggested in this thesis.

C. α7 Integrin Transgenic Mice are Protected from Functional Deficits Post-Injury

A single bout of eccentric exercise can disrupt structures within the excitation-contraction (E-C) coupling complex and induce ultrastructural damage within Z bands, resulting in significant deficits in force production for several weeks. In this study, a trend for a decrease in force was observed in WT mice at 7-days PE, reflective of muscle injury. In contrast, muscle force was not only preserved in α7Tg mice, reflective of protection from injury, but a trend towards an increase in force was observed at 7-days PE. An improvement in muscular function from a single bout of exercise is not typically observed. However, the rapid increase in number of muscle fibers may provide the ability to generate more tension.

D. α7 Integrin Transgenic Mice Have Larger Muscle Fibers

To determine if the preservation in force was due to the presence of *de novo* fibers or individual muscle fiber hypertrophy in the α7Tg mice, cross sectional areas (CSA) of the muscle fibers were evaluated. A trend towards an increase in CSA was observed in both the WT and α7Tg mice from basal to 7-days PE. Interestingly, the α7Tg mice have a trend towards slightly larger fiber CSAs than the WT mice. Therefore, the increase in force seen in the α7Tg animals is likely due to both increased myogenesis and enlargement of existing or regenerating fibers.

E. Conclusion

In conclusion, this study demonstrates that exposure to a single bout of downhill treadmill running exercise induces myogenesis in both WT and α7Tg transgenic mice. While myogenesis is present in both groups, it is significantly accelerated in α7Tg mice. This may be due to protection from injury, recruitment of MSCs, activation of satellite cells, modulation of the extracellular environment or a combination of these factors. The exploration of integrin kinetics *in vivo* and integrin-mediated myogenesis is in its infancy. The studies provided in this thesis have provided the first steps toward understanding the mechanistic basis for α7 integrin-mediated increases in muscle growth and function.

Given the findings of this study, I believe α7 integrin expression to be a highly promising therapeutic tool in the quest for the prevention of exercise-induced injury, disease-related muscle atrophy, and age-related deficits in muscle function. Gene therapy or pharmaceutical agonists which upregulate the α7 integrin may be used alone or in combination with stem cell therapy to enhance myogenesis.

The potential applications of this research are great and suggest further research in the field to expand on the currently limited knowledge of the myogenic response of the α7 integrin to exercise-induced strain. Specific myogenic capabilities of the Sca-1+ CD45- MSCs are also anxiously awaited in order to disconnect the α7 integrin from the MSCs, in the determination if one or both factors are truly responsible for the significant increases in myogenesis, strength, and hypertrophy observed in this study.

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APPENDIX A: EXPANDED METHODS

Appendix A.1 eMHC(47A)/Sca-1 double stain IHC

Procedure	Details
Prepare Samples	Make10 um sections
Fix	Acetone:5 min, apply Immunopen
Wash	1 X PBS: 5 min, 3X
Block	10% Horse Serum diluted in 1X PBS: 20 min Blocking Fabs 1:20 dilution in 10% HS: 30 min
Wash	2% HS diluted in 1X PBS: 5 min, 3X
1st Primary Antibody	47A 1:10 dilution in 1% HS: 1 hour
Wash	1% HS diluted in 1X PBS: 5 min, 3X
2nd Primary Antibody	SCA-1 1:100 dilution in 1%HS: 1 hour
Wash	1% HS diluted in 1X PBS: 5 min, 3X
1st Secondary Antibody	(in dark) FITC anti-mouse 1:100 dilution in 1% HS: 1 hour
Wash	1% HS diluted in 1X PBS: 5 min, 3X
2nd Secondary Antibody	(in dark) TRITC anti-Rat 1:100 dilution in 1% HS): 1 hour
Wash	1% HS diluted in 1X PBS): 5 min, 3X
Coverslip	1 drop Vectashield w/ DAPI over each slide Apply coverslip Seal with clear nail polish Store in refrigerator for up to approx. 6 months

Appendix A.2 α7/Sca-1 double stain IHC

Procedure	Details
Prepare Samples	Make 10 um sections
Fix	Acetone: 5 min, apply Immunopen
Wash	1 X PBS: 5 min, 3X
Block	5% BSA diluted in 1X PBS: 20 min Block Fabs 1:20 dilution in 5% BSA: 30 min
Wash	1% BSA diluted in 1X PBS: 5 min, 3X
1st Primary Antibody	α7B 1:500 dilution in 1% BSA: 1 hour
Wash	1% BSA diluted in 1X PBS: 5 min, 3X
2nd Primary Antibody	SCA-1 1:100 dilution in 1%BSA: 1 hour
Wash	1% BSA diluted in 1X PBS): 5 min, 3X
1st Secondary Antibody	(in dark) FITC DaRb 1:100 dilution in 1% BSA:1 hour
Wash	1% BSA diluted in 1X PBS: 5 min, 3X
2nd Secondary Antibody	(in dark) TRITC anti-Rat 1:100 dilution in 1% BSA: 1 hour
Wash	1% BSA diluted in 1X PBS: 5 min, 3X
Coverslip	1 drop Vectashield w/ DAPI over each slide Apply coverslip Seal with clear nail polish Store in refrigerator for up to approx. 6 months

Appendix A.3 Pax7 IHC

Procedure	Details
Prepare Samples	Make 10 um sections
Fix	Acetone: 5 min, apply Immunopen
Wash	1 X PBS: 5 min, 3X
Block	10% Horse Serum diluted in 1X PBS: 20 min Blocking Fabs 1:20 dilution in 10% HS: 30 min
Wash	2% HS diluted in 1X PBS): 5 min, 3X
1st Primary Antibody	Pax7 1:200 dilution in 1% HS: 1 hour
Wash	1% HS diluted in 1X PBS: 5 min, 3X
Secondary Antibody	(in dark) FITC anti-mouse 1:100 dilution in 1% HS:1 hour
Wash	1% HS diluted in 1X PBS: 5 min, 3X
Coverslip	1 drop Vectashield w/ DAPI over each slide Apply coverslip Seal with clear nail polish Store in refrigerator for up to approx. 6 months

Appendix A.4 H&E Staining Protocol for Automatic Stainer

Procedure	Details
Prepare Samples	Make 10 um sections
Fix	100% Acetone: 5 min at -20°C
Wash	Place in running water for 5 minutes
Hematoxylin	Add to Gill's Hematoxylin for 3 minutes
Wash	Place in running water for 5 minutes
Scott's Solution	Place in Scott's solution for 3 minutes
Wash	Place in running water for 5 minutes
Eosin	Add to Eosin and rock for 1 min 30 sec. (dilute Eosin to 1X with 70% EtOH before staining)
Rinse	Dip in 70% EtOH for 30 sec.
Rinse	Dip in 95% EtOH for 30 sec.
EtOH	Place in 100% EtOH for 2 minutes
Xylene	Clear in Xylene for 5 minutes
Coverslip	Mount with Permount and coverslip

APPENDIX B: RECORD OF DATA

Appendix B.1 eMHC Raw Data

B WT	B TG	2D WT	2D TG	4D WT	4D TG	7D WT	7D TG
T42: 17	T35: 30	T41: 34	T52: 123	T48: 61	T49: 103	T46: 43	T40: 84
T42: 23	T35: 24	T41: 9	T52: 63	T48: 38	T49: 166	T46: 84	T40: 111
T42: 13	T35: 18	T41: 19	T52: 178	T48: 56	T49: 61	T46: 80	T40: 24
T43: 12	T36: 32	T55: 16	T53: 41	T27: 8	T50: 107	T45: 36	T54: 148
T43: 13	T36: 24	T55: 10	T53: 32	T27: 5	T50: 32	T45: 44	T54: 76
T43: 11	T36: 33	T55: 19	T53: 41	T27: 6	T50: 28	T45: 34	T54: 89
T44: 10	T37: 24	T30: 4	T56: 55	T73: 34	T25: 10	T19: 62	T57: 65
T44: 5	T37: 33	T30: 9	T56: 46	T73: 26	T25: 8	T19: 58	T57: 113
T44: 9	T37: 33	T30: 8	T56: 46	T73: 22	T25: 5	T19: 69	T39: 69
T16: 1	T38: 21	T71: 7	T28: 116	T74: 33	T26: 84	T18: 5	T39: 40
T16: 2	T38: 15	T71: 14	T28: 86	T74: 40	T26: 78	T18: 9	T39: 78
T16: 1	T38: 10	T71: 6	T28: 58	T74: 59	T26: 97	T18: 4	T5: 9
T61: 0	T3: 0	T72: 18	T32: 32	T75: 61	T33: 41	T64: 50	T5: 5
T61: 2	T3: 1	T72: 10	T32: 49	T75: 24	T33: 38	T64: 41	T5: 10
T61: 4	T3: 2	T72: 12	T32: 38	T75: 37	T33: 27	T64: 41	T21: 4
T78: 0	T12: 7	T76: 6	T34: 2	T77: 35		T66: 57	T21: 1
T78: 5	T12: 6	T76: 7	T34: 1	T77: 29		T66: 61	T21: 4
T78: 3	T12: 6	T76: 19	T34: 0	T77: 42		T66: 53	T20: 8
	T58: 2						T20: 3
	T58: 4						T20: 5
	T58: 4						
	T59: 5						
	T59: 3						
	T59: 3						
	T60: 9						
	T60: 15						
	T60: 11						

Appendix B.2 CLN Raw Data

B WT	B TG	2D WT	2D TG	4D WT	4D TG	7D WT	7D TG
T42: 20	T35: 16	T41: 33	T52: 126	T48: 27	T47: 79	T46: 35	T40: 28
T43: 16	T36: 18	T55: 29	T53: 86	T27: 20	T49: 68	T45: 32	T54: 73
T44: 24	T37: 17	T30: 14	T56: 86	T73: 21	T50: 86	T19: 61	T57: 89
T16: 5	T38: 25	T71: 21	T28: 80	T74: 25	T25: 22	T18: 47	T39: 85
T61: 12	T3: 5	T72: 17	T34: 30	T75: 24	T33: 17	T64: 34	T21: 14
T78: 12	T12: 10	T76: 28	T32: 59	T77: 15	T26: 21	T66: 49	T20: 12
	T58: 14						T5: 73
	T59: 13						T4: 15
	T60: 13						

Appendix B.3 Pax7 Raw Data (Injected Cell Samples)

	Basal Injected	Basal Saline	Exercised Injected	Exercised Saline
1	65	70	322	104
2	51	60	161	111
3	57	51	171	76
4	60	59	173	33
5	51	60	141	30
6	52	51	110	36
7	27	70	113	39
8	60	69	114	67
9	54	54	153	65
10	76	75	148	32
11	83	61	89	39
12	148	71	89	35
13	175	96	87	39
14	51	97	77	30
15	59	78	166	27
16	49	63	98	39
17	112	56	67	39
18	69	50	57	37
19	68	52	64	34
20	51	56	94	37
21	52	67	70	34
22	69	64	84	51
23	62	51	66	52
24	54	47	48	43
25	73	54	42	49
26	65	46	101	59
27	51	69	51	35
28	57	48	52	38
29	76	51	66	72
30	67	74	101	55
31	87	77	99	57
32	102	60	105	56
33	68	65	115	46
34	69	67	169	36
35	67	74	162	61
36	82	89	158	35
37	67	66	120	42
38	96	51	86	39
39	86	56	81	38
40	80	58	180	63
Totals	2848	2533	4450	1910

Appendix B.4 Force Measurement Raw Data

Animal	Group	BW	MW	Rep 1	Rep1/MW	Ave	SE
BX2 23/A 1FR	TG/EX	19.8	0.133	27.51	206.8421053		
BX2 23/A 2FL	TG/EX	22.89	0.144	31.43	218.2638889		
SBX 2/C 5FR	TG/EX	18.32	0.0982	33.43	340.4276986		
SBX 2/C 6FL	TG/EX	18.41	0.0902	18.47	204.767184	242.5752	32.75216
BX2 22/A 3FRL	TG/B	19.8	0.133	25.05	188.3458647		
BX2 22/A 4FT	TG/B	20.15	0.139	23.89	171.8705036		
BX2 25/A 2FL	TG/B	19.86	0.149	24.6	165.1006711		
SBX 2/C 1FR	TG/B	16.56	0.0875	16.03	183.2	177.1293	5.283682
BX2 25/A 1FR	WT/EX	19.7	0.182	24.9	136.8131868		
BXS 1/D 5FL	WT/EX	18.87	0.116	21.87	188.5344828	162.6738	25.86065
BX2 22/A 3FRL	WT/B	18.72	0.137	33.46	244.2335766		
SBX 3/A 6FRL	WT/B	19.05	0.1092	24.27	222.2527473		
BXS 1/A 4FR	WT/B	17.06	0.0892	18.5	207.3991031		
SBX 2/B 1FR	WT/B	18.3	0.1078	20.62	191.2801484	216.2914	11.2581

Appendix B.5 Pax7 T-Sample Raw Data

T61 B WT	T58 B TG	T66 7D WT	T64 7D WT	T78 B TG	T59 B WT	T60 B TG	T71 2D WT	T72 2D WT	T76 2D WT	T73 4D WT	T74 4D WT	T75 4D WT	T77 4D WT	T39 7D TG	T45 7D WT
78	34	70	70	89	67	115	88	133	40	45	30	101	141	30	49
92	43	68	39	88	74	69	78	133	52	51	28	77	92	17	87
77	47	38	29	35	96	76	138	96	79	46	41	96	56	7	77
39	44	61	24	55	85	45	64	94	59	48	22	48	58	22	52
23	39	38	36	48	67	66	62	63	57	70	27	33	53	35	47
45	29	26	30	51	77	43	53	83	54	61	36	30	46	26	35
76	30	34	47	33	85	57	45	109	58	52	30	37	77	24	46
51	82	42	36	47	38	68	58	98	70	42	66	68	152	18	64
53	61	36	24	46	26	53	94	78	120	50	32	65	154	46	60
55	55	32	28	54	55	105	70	54	83	63	57	38	70	33	81
30	31	65	18	44	52	73	56	60	73	54	46	40	39	19	44
23	42	62	14	43	48	67	59	72	55	33	25	14	46	29	33
61	48	56	23	45	39	53	60	48	62	44	35	17	30	38	42
48	32	35	11	49	47	166	63	46	66	48	43	23	54	29	27
69	31	24	28	39	7	135	59	45	70	47	27	35	57	32	26
20	33	14	35	40	5	45	96	50	96	58	42	20	78	22	35
38	49	35	47	52	19	53	65	71	108	69	41	12	122	20	59
48	59	30	17	26	3	73	65	53	77	34	36	15	97	48	44
44	25	33	25	25	36	41	67	50	63	29	58	4	84	32	50
49	46	34	15	40	42	62	36	69	41	61	40	13	48	13	36
48	47	56	14	51	45	40	42	75	92	92	18	42	46	14	45
32	51	5	19	43	61	44	40	30	69	51	30	49	80	67	40
29	81	33	29	30	44	60	43	41	69	36	23	46	38	47	57
25	54	78	41	35	56	70	47	48	54	34	19	74	55	18	72
23	52	80	19	28	51	54	43	30	64	44	36	67	94	18	46
27	8	50	15	33	36	72	35	41	49	49	32	67	83	12	44
35	20	22	6	39	66	102	50	39	44	66	28	68	136	17	42
36	32	7	7	51	43	101	43	47	48	36	45	40	58	32	42
44	66	7	18	43	9	90	24	43	58	30	44	52	27	15	45
33	44	32	12	33	44	53	23	45	82	27	38	80	31	45	37
42	62	60	22	48	22	98	42	52	68	35	23	62	47	31	42
4	24	38	49	24	3	101	44	65	37	30	45	67	59	22	47
11	51	40	2	20	10	90	100	52	51	49	45	48	71	45	44
18	47	57	5	52	5	62	111	97	59	55	45	27	30	39	36
39	33	6	66	29	6	59	70	53	31	29	71	14	23	62	65
20	51	4	41	42	4	59	82	61	47	27	36	32	22	47	48
18	49	11	12	44	3	111	136	46	89	42	37	59	34	47	45
21	24	39	9	54	3	133	75	54	83	49	47	37	46	44	52
87	25	43	2	83	23	64	58	58	47	38	30	6	65	57	48
52	34	53	4	48	6	68	67	49	37	28	27	5	57	66	45

T54 7D TG	T57 7D TG	T53 2D TG	T55 2D WT	T56 2D TG	T49 4D TG	T50 4D TG	T36 B TG	T43 B WT	T40 7D TG	T46 7D WT	T35 B WT	T52 2D TG	T26 4D TG	T33 4D TG	T4 2D TG
100	44	62	105	54	46	70	30	39	89	43	22	188	36	71	118
49	32	54	36	66	77	35	40	35	90	41	19	159	39	61	83
47	37	51	43	41	47	50	49	46	72	28	13	104	44	70	54
32	21	31	55	39	71	62	58	37	31	25	14	68	36	46	9
15	18	43	91	47	49	67	36	47	50	48	3	68	34	63	60
47	50	52	84	28	64	53	41	37	32	30	10	66	56	36	115
43	48	33	53	39	46	51	52	46	38	43	3	99	39	36	92
24	20	40	25	40	41	26	50	66	30	50	7	59	39	20	55
20	17	47	25	44	43	18	34	47	50	40	8	57	45	9	78
38	15	60	25	33	39	28	26	37	58	35	11	52	65	51	51
34	39	86	40	25	51	22	40	39	36	58	36	40	42	32	6
20	31	32	16	42	62	23	42	32	32	39	50	39	53	67	1
24	34	61	15	44	61	46	41	39	37	64	20	39	31	52	7
28	33	74	20	39	63	70	35	32	24	58	15	67	45	76	3
20	42	79	14	45	62	59	41	47	47	54	2	97	37	110	8
22	33	60	21	36	53	73	27	26	37	51	3	73	30	123	33
35	38	48	38	47	42	60	28	35	35	54	9	51	42	84	5
35	50	70	57	47	28	46	17	37	50	57	12	40	15	66	1
26	34	89	69	60	35	32	28	29	60	73	18	40	6	46	0
31	64	77	111	38	60	31	19	21	45	42	20	58	23	42	0
24	69	83	58	50	67	28	31	44	32	44	25	56	6	60	5
22	36	44	30	41	45	19	28	40	45	48	19	37	13	34	4
23	31	105	26	18	36	29	52	43	30	47	20	48	3	47	12
22	50	79	14	152	50	47	21	55	51	47	19	96	1	50	7
26	63	68	21	109	36	42	33	45	42	43	29	96	5	49	68
33	57	45	21	127	37	36	44	50	36	33	42	65	2	76	121
36	51	55	28	118	30	84	46	84	73	37	22	38	7	53	63
36	40	49	13	106	34	44	42	93	40	27	18	64	2	41	54
44	48	50	19	59	38	34	46	58	39	35	21	19	6	63	93
34	29	35	22	64	106	22	35	43	31	40	21	14	2	58	100
32	37	20	84	56	83	32	73	40	38	38	23	60	2	72	6
14	55	21	26	53	76	27	64	51	55	46	37	80	3	56	0
44	28	59	41	86	42	31	17	27	36	33	33	21	47	76	2
31	34	21	33	61	50	42	24	76	23	33	34	12	53	66	46
57	47	17	14	66	50	29	48	42	37	35	11	37	22	38	84
31	21	43	54	52	56	39	53	62	43	28	4	78	12	75	53
27	37	59	34	79	94	35	30	54	36	33	3	64	15	98	1
37	28	30	55	70	98	35	63	42	58	32	8	60	10	43	0
32	29	46	60	72	99	43	57	33	52	39	18	60	66	62	0
27	35	53	24	45	72	65	24	19	42	44	16	22	67	47	2